Eesti Maaülikool
Estonian University of Life Sciences

FACTORS INFLUENCING THE DISTRIBUTION AND OVERWINTERING SURVIVAL OF THE POTATO ROT NEMATODE (*Ditylenchus destructor* Thorne 1945)

KARTULIINGERJA
(*Ditylenchus destructor* Thorne 1945)
LEVIKUT JA TALVIST ELLUJÄÄMIST MÕJUTAVAD FAKTORID

EHA ŠVILPONIS

A Thesis
for applying for the degree of Doctor of Philosophy
in Agricultural Sciences in Plant Protection

Väitekiri
Filosoofiadoktori kraadi taotlemiseks
Põllumajanduse õppekava taimekaitse erialal

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LIST OF ORIGINAL PUBLICATIONS

The current thesis is a review of the following papers which are referred to by Roma n numerals in the text. The papers are reproduced by kind permission of the following journals: Zemdirbyste-Agriculture (II), Entomologia Experimentalis et Applicata (III, IV), Nematology (V).

I **Švilponis, E.** Impact of trade on distribution of potato rot nematode (*Ditylenchus destructor*) and other plant nematodes. Agronomy Research (submitted).

II **Švilponis, E.**; Luik, A.; Krall, E. (2008) Plant parasitic ditylenchids in Estonia. Zemdirbyste-Agriculture, 95(3), 186 - 193.

III Hiiesaar, K.; Williams, I.; Luik, A.; Metspalu, L.; Muljar, R.; Jõgar, K.; Karise, R.; Mänd, M.; **Svilponis, E.**; Ploomi, A. (2009) Factors affecting cold hardiness in the small striped flea beetle, *Phyllotreta undulata*. Entomologia Experimentalis et Applicata, 131: 278–285.

IV Hiiesaar, K.; Williams, I.H.; Mänd, M.; Luik, A.; Jõgar, K.; Metspalu, L.; **Švilponis, E.**; Ploomi, A.; Kivimägi, I. (2011) Supercooling ability and cold hardiness of the pollen beetle. Entomologia Experimentalis et Applicata, 138(2), 117 - 127.

V **Švilponis, E**.; Hiiesaar, K.; Kaart, T.; Metspalu, L.; Mänd, M.; Ploomi, A.; Williams, I.H.; Luik, A. (2011) Low temperature survival of the potato rot nematode *Ditylenchus destructor* Thorne (Tylenchida: Anguinidae) post-eclosion stages. Nematology, 13(8), 967-973.

VI **Švilponis, E.**; Hiiesaar, K.; Metspalu, L., Mänd, M., Jõgar, K.; Veromann, E., Luik, A. (2011) Snow manipulation as an alternative nematode control strategy. Zemdirbyste-Agriculture (submitted).

Table 1. Author's contribution to each article (%).

	I	II	III	IV	V	VI
Idea and design	85	80	10	10	75	95
Data collection	100	95	5	5	100	80
Data analysis, statistics	100	100	5	5	45	95
Writing	95	95	5	5	85	95

ABBREVIATIONS

LLT	Lower Lethal Temperature (temperature at which 100% of the population is killed)
LT50	Lethal Temperature to 50% mortality (temperature at which 50% of the population is killed)
LT90	Lethal Temperature to 90% mortality (temperature at which 90% if the population is killed)
SCP	Supercooling Point
EPPO	European and Mediterranean Plant Protection Organization

1. INTRODUCTION

Potato rot nematode, *Ditylenchus destructor* Thorne 1945 was a serious pest of potato in Europe in 1950 – 1970s. During that period, it was evaluated to cause greater crop losses than potato cyst nematode, *Globodera rostochiensis* in Estonia (Krall, 1974). The problem in Estonia was eventually alleviated by using in-vitro cultured basic material, informing state farms' agronomists and seed potato producers of potential threats and by changing the varieties to more resistant or tolerant ones.

In the middle of 1990s, neither stem nor potato rot nematodes were considered among 55 major crop pests and 50 diseases for the annual national prognosis survey (Taim & Soobik, 1994). By that time, 100% of super elite seed potatoes originated from in vitro clone cultures; but since 1994, the national seed potato micropropagation laboratory EVIKA lost the possibility to multiply the protected varieties, which lead to sudden decrease of the certified seed potato production area from 1300 ha to 76 ha in 1999 (Rosenberg, 2002). Ever since, increasing concerns have been raised due to the potato rot nematode in Estonia as well as in several other potato growing regions.

There have been several cases of culling seed potato lots due to infestation by *D. destructor* in recent years in Estonia. Relevant producers lose their annual income as well as initial investments since they cannot market the contaminated yield. Ware potato producers are advised by national inspectors not to grow potatoes in their infested fields. Random ware potato checks have resulted in establishing infestation with potato rot nematode in 1– 8% of analyzed samples as a side-product of bacterial disease monitoring since 2006. Scarce status reports give but a little substance for convincing conclusions about the gravity of the current problem. Therefore a review on international experience and survey of the most recent interception reports together with determination of factors influencing the distribution and invasiveness of the species could provide valuable information.

In Northern temperate regions, overwintering soil-dwelling invertebrates may encounter severe conditions since almost every year short periods with extremely low temperatures occur. Despite the severity of the winter, pest populations remain continuously high. Even though there have been

no recent studies on potato rot nematode behaviour in cropping systems, there is reason to assume that at least a part of the population must be adapted to survive the range of minimum temperatures it encounters in the microhabitat. Differences in ecological flexibility of various life stages of nematodes may be expected, but apart from encapsulated (pre-eclosion) stages, so far there has been little solid evidence in plant nematodes.

Invertebrate cold tolerance has been studied mainly in insects or other arthropods. Scientists of the laboratory of experimental entomology of the former Institute of Zoology and Botany of the Estonian Academy of Sciences, currently employed by the Estonian University of Life Sciences have long expertise in physiological research, including measurement of insect cold tolerance and supercooling (Merivee, 1970, 1978; Kuusik, 1971, Kuusik *et al.,* 1995, Metspalu *et al., 2*003). The equipment had been developed and adapted for measuring primarily the cold hardiness to short exposures in large insects (noctuid moths, carabid beetles, pollinating insects etc.). However, so far there was no technology to measure long exposures in smaller subjects. In the beginning of the current research, new equipment (liquid thermostat, chromel-alumel thermocouples, data loggers and the relevant software) was obtained to address new research questions. Prior to conducting the research with new apparatus on nematodes, testing was carried out using insects to facilitate development of general practices as well as manipulation skills. To develop laboratory techniques and methods most suited for application in the main research with the potato rot nematode, flea beetles (*Phyllotreta undulata*) and pollen beetles (*Meligethes aeneus*) were used in preliminary tests. Reasons for preferring those species over other organisms targeted by the same research group were their relatively small body mass as well as their soil-dwelling habits for overwintering.

A complex of control measures including a general detection survey of the potato rot nematode, practice of crop rotation with a potato-free period, pest free seed potato, and weed control has been recommended to avoid the economic consequences due to the pest (Kikas, 1969). Crop rotation in 3–4 years with cereals and maize together with weed control has been foundto be exceptionally effective (Sturhan & Brzeski, 1991). Nevertheless, barley and wheat together with many wild plants and common weed species are reported to belong in the host range of the potato rot nematode (Sturhan & Brzeski, 1991).

There have been instances with contaminations of pedigree seed potato nuclear stock occurring at least 5 years after last potato crop grown in the soil. Contradicting evidence on survival ability of the *D. destructor* in soil may easily lead to excessive restrictions in land usage and does not motivate producers to cooperate with the national authorities in the establishment of pest free production sites. Information about interactions between air temperatures and snow cover to resulting soil temperatures and survival of soil-dwelling macroinvertebrates, would prove useful for developing novel knowledge-based pest control strategies.

2. REVIEW OF THE LITERATURE

2.1. Dry rot of potatoes and an invertebrate pathogen – Potato rot nematode *Ditylenchus destructor* as the causal agent

Dry rot of potatoes is a storage disorder caused a.o. by a polyphagous pest, the potato rot nematode, *Ditylenchus destructor* Thorne 1945. The disease has resulted in serious economic damage, especially, in Eastern European potato production. Stem nematodes of the genus *Ditylenchus* hatch from eggs as vermiform second-stage juveniles (J2); the first moult precedes eclosion. The juvenile grows and undergoes three more moults (J3, J4 and adult) to become a mature male or female. *D. destructor* is a migratory endoparasite mainly on underground organs.

The potato rot nematode has become important as a pest of potatoes at temperatures of 15– 20 °C and at a relative humidity above 90% (Sturhan & Brzeski, 1991, CAB International, 2010). However, according to Ilyashenka and Ivaniuk (2008), in Belarus, potato rot nematode has been favoured by hot dry weather and moisture deficiency in the soil. Likewise, in 1993, *D. destructor* was reported to be present both in cold and warm regions of Albania, with infestation levels of 3.5– 12% (EPPO, 1995). The damage threshold of *D. destructor* is 20– 50 individuals / 1 kg of soil (Butorina *et al., 2*006). Similarly, serious damage by *Ditylenchus dipsaci* (Kühn, 1857) Filipjev, 1936 in onion, rye and other crops may be expected at low densities of 10 nematodes / 500 g of soil (Seinhorst, 1956; Sturhan & Brzeski, 1991).

Andersson (1971) states that, even in heavily infested fields, populations of this nematode can be eradicated or decreased if the field is kept free from biennial or perennial host plants for a few years. Nevertheless, in current agriculture and horticulture, weeds may play a desirable role, providing diversity, ecosystem functions and supportfor many other species (Marshall *et al., 2*003). *D. destructor* has a very wide range of host plants and fungi, which is why this pest presents a serious threat in newly cultivated fields with an unrecorded cropping history, especially if reduced tillage is practised. A positive relationship between less-intensive soil management practices and the incidence of herbivorous nematodes has been established by several experiments (Thomas, 1977; Hendrix *et al.,* 1986; Hou *et al., 2*010). Thus, potato rot nematode may be an

increasing problem in contemporary agriculture (**II**) even though good phytosanitary practices in seed potato production would greatly assist in escaping economic damage.

2.1.1. Invasion and spread of *D. destructor* (I)

The wide geographic range of Ditylenchids (Fig. 1) has been interpreted as evidence of evolutionally ancient origin of the taxonomic group (Sturhan & Brzeski, 1991).

Nevertheless, many countries with the agricultural sector of major importance in the economy have targeted ditylenchid nematodes by legislative measures. According to Lehman (2004), this trend has been increasing as *D. dipsaci* was reported to be regulated in 23 countries in 1982 while by 2000 the number had doubled – 58. The same data for *D. destructor* are 12 and 53, respectively. Hockland and her co-workers suggest this is because these species are easily transported by the international plant trade due to their endoparasitic nature (Hockland *et al., 2*006).

Figure 1. The distribution of potato rot nematode *Ditylenchus destructor* in Europe. Source: Fauna Europaea, 2004. www.faunaeur.org

ProMED (electronic reporting system for outbreaks of emerging infectious diseases and toxins) reports of plant diseases published between 1996 and 2002 show that nematodes are only minor pathogens causing about 1% of the plant emerging infectious diseases (Anderson *et al., 2*004). It was assumed that this reflects the proportion of targeted research interest rather than the actual proportion established by biodiversity inventories. Similarly, it has been noted that the number of literature citations on *Ditylenchus* species has been drastically restricted (Sturhan & Brzeski, 1991).

Apart from general quarantine regulations, there are no intergovernmental programs targeted against the potato rot nematode in Europe. The total financial contribution granted in the framework of the plant health solidarity regime for the years 1999– 2009 in the European Union was approximately € 30 million. The main beneficiary of the regime to date has been Portugal, with a total maximum EC contribution of 73% of total solidarity funding allocated for an eradication campaign against the pinewood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer, 1934) (Christodoulou, 2010). Additionally, five EU member states (Czech Republic, Hungary, Poland, Sweden and Slovenia) have undertaken control measures against the potato cyst nematodes *Globodera rostochiensis* and *G. pallida* totalling a financial contribution of € 3,800 from the solidarity funding. Yet, there is little evidence on allocations for actions on the potato rot nematode.

After the species differentiation in 1945, *D. destructor* was recorded in many countries, mostly from temperate regions (Sturhan & Brzeski, 1991). According to EPPO (2007), potato rot nematode is reported to be present in over 70% of the member countries of the organization in the European territories (Fig. 2). Most countries report the pest status as being of limited distribution or with only a few published records.

Fluctuation of potato rot nematode population density is proposed by Gul'skova (2006). She reports the peak of potato rot nematode damage in the former Soviet Union occurring in the 1960– 1970s. Nowadays, in Lithuania, *D. destructor* has been repeatedly intercepted in seed potato. In 1999, it was discovered on seed potatoes from 12 sites (EPPO, 2000); in the following year, 25 outbreaks of *D. destructor* were reported in 1258 tons of domestic seed potatoes (22 cultivars) (EPPO, 2001) and in 2002, 24 outbreaks were found in potato farms, with 17 new outbreaks and

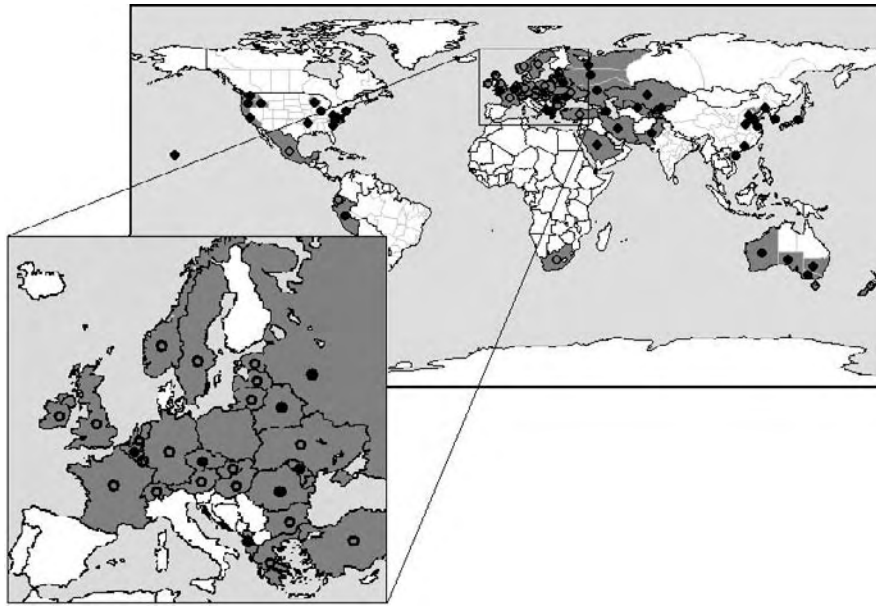

Figure 2. The distribution of potato rot nematode *Ditylenchus destructor*. Full circles demarcate presence in national or sub-national level, open circles demarcate presence only in some areas. Source: European and Mediterranean Plant Protection Organization, Distribution maps of quarantine pests for Europe, 2006. www.eppo.org .

628 tons of potatoes declared infested by the nematode, including 477 tons of seed potatoes (EPPO, 2003). A year later, three more outbreaks were located in seed potato-growing farms (EPPO, 2004). In 2005, the nematode was detected in 33 samples of seed potatoes (942 tons and 8 farms) and in 48 samples of ware potatoes (894 tons, 26 farms) (EPPO, 2005).

In Belarus, *D. destructor* is unevenly distributed and occurs mainly in the region of Minsk (Ilyashenka & Ivaniuk, 2008). In the Minsk region, 69% of all examined lots of seed potatoes were found infested, whereas in the Brest region this index was 26; in Gomel region 29; Mogilev 30 and in Vitebsk 33%. By the end of the first decade of the 21.th century, potato dry rot, caused by two stem nematode species – *D. destructor* and *D. dipsaci* – became widespread and tuber infection by the nematodes sharply decreased seed and food potato quality. Yield losses because of potato infection by nematodes can be 30-80% (Ivaniuk *et al., 2*008).

Generally, plant and soil community assembly during secondary succession depends on the initial species composition, colonization from the local species pools, and on the response of the resident and colonizing species to the changing biotic and abiotic environmental conditions as shown by Kardol (2007). His data indicated that changes in the soil nematode community composition were mainly due to gradual shifts in dominance patterns in response to altered environmental conditions, even though, clear successional trends in densities of endoparasitic plant-feeding nematodes could not be determined. Polish researchers have found *D. destructor* present on dicotyledonous weed species *Anthemis arvensis*, *Bertoroa incana*, *Lycopsis arvensis* in weed samples when 205 fields of common crops (20% of which were fallow) were surveyed in Wielkopolska region during 1993– 1994 (Kornobis & Wolny, 1997, as cited in EPPO, 1998).

The pan-European Invasive Alien Species inventory established by the EC funded project DAISIE enlists *D. destructor* as alien for the majority of European countries (Fig. 3).

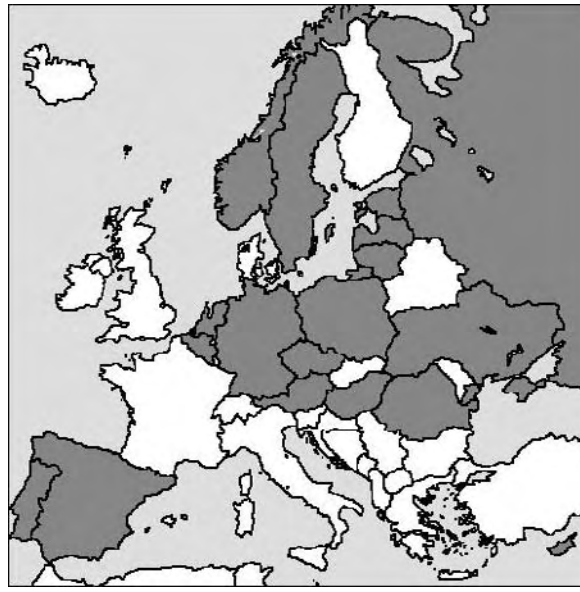

Figure 3. The distribution of potato rot nematode *Ditylenchus destructor* in Europe. Source: Pan-European Invasive Alien Species inventory, project DAISIE, 2003. www.europe-aliens.org.

Potato rot nematode may spread not only by the material planted but also via irrigation water. It has been shown that, despite the general tendency to cause damage in colder and more humid areas (EPPO/CABI, 1997), *D. destructor* has been detected in the Iranian arid or semi-arid provinces of Semnan and Tehran, where the potato production relies heavily on irrigation. The percentage of infestation with the nematodes causing various root lesions was 87% and 54% in Semnan and Tehran, respectively (Maafi *et al., 2*004). The percentage of field infestation to *D. destructor* was 11% in Semnan and 2% in Tehran samples (Moafi *et al., 2*005). Similarily, *Ditylenchus sp.* has been found infesting 28% of the samples from six major potato-producing regions in Saudi Arabia in the survey conducted from 1989– 1991 (Al Hazmi *et al., 1*993, as cited by EPPO, 1994).

In the UK, according to a database of 325 non-native invertebrate plant pests, established in Great Britain between 1787 and 2004, it was recognized that 47% of all establishments occurred after 1970, with all but one of the significant post– 1970 establishments on ornamental plants (Christodoulou, 2010). In Japan, *D. destructor* has been reported to be causing damage to 18 host plants including *Brassica chinensis*, *B. oleracea*, *Capsicum annuum*, *Dendranthema morifolium*, *Cucumis sativus*, *Cucurbita moschata* and *Lycopersicon esculentum* (Nakanishi, 1979). However, serious problems occur essentially on iris and garlic crops, spreading despite the control measures (Nishizawa, 1999 as cited in EPPO, 1999). In China, it was first found parasitizing sweet potato, potato and Mentha (Ding & Lin, 1982). From 2004 to 2006, a root rot was observed on American ginseng (*Panax quinquefolium*) cultivated in the Beijing area and in Hebei Province in China. Pathogenicity tests confirmed that *D. destructor* was causing the disease (Zhang & Zhang, 2007). Ginseng was also found infested in Korea (Young & Seung, 1995)

Conversely, the general quarantine regulations against the potato rot nematode in European territories have been abolished, as it was removed from EPPO A2 quarantine pest list in 1984, because of its very wide distribution in the region (EPPO, 1987, 1988). Nowadays, *D. destructor* is regulated under the EU Plant Health Directive (2000/29/EC) Annex II/A2, e.g., it is enlisted among harmful organisms whose introduction into, and whose spread within, all member states shall be banned if they are present on flower bulbs and corms of *Crocus*, *Hyacinthus*, *Iris*, *Trigridia*, *Tulipa*, miniature cultivars and their hybrids of the genus

Gladiolus, intended for planting, and potato tubers (*Solanum tuberosum*), intended for planting. Due to these rules, the findings of *D. destructor* on other ornamental host species are not subjected to notification nor are they actionable.

2.1.2. Significance of *D. destructor* in Estonia (II)

In Estonia, nematological research and national surveillance was begun in 1960– 1970s due to serious economic damage by potato nematodes. Krall (1959) reported regular 6–7% infestation of potato rot nematode in storage and suggests the pest was widely distributed in the continental part of Estonia already in 1953 when first notifications of pest damage appeared. Kikas (1969) illustrates the severity of the pest by examples from the Tõrva region where about 50% of the tuber crop was infested by the end of the growing season after healthy (symptomless) seed had been planted on a possibly contaminated soil. Next year there were 80–90% tubers infested on the same plot planted with tubers from the previous yield.

Mass and single clone selection and indexing have been systematically employed as quality measures in Estonian seed potato production due to the possibility of export since the 1930s (Võsaste, 1971). Production experiments for potato disease eradication were started at the Estonian Research Institute of Agriculture by E. Kaarep in 1958 (Jaanvärk, 1966), although in early years mainly fungal diseases and potato viruses were targeted. Sinijärv (1971) has discussed how potato rot nematodes were distributed by means of contaminated seed potatoes. Due to higher procurement price of 'Jõgeva Kollane', this table potato variety production was forced. Abnormal demand and poor supply created a market for low-quality seed. Sinijärv (1971) admits that all producers of 'Jõgeva Kollane' elite seed had soils infested with potato rot nematode and recommended the use of pest-free fields for seed production.

The National Plant Protection Board established the distribution level of potato rot nematode to be 50% based on the seed potato quality analyses in spring 1970 (Randalu, 1971). From 1973, there are records on the regular work of the certification commission for seed potato super elite and elite categories (Rosenberg, 2002). A new seed potato production scheme was enforced by the Agricultural Industry Assembly to use only in vitro basic material in 1984 and 100% of super elite originated from

in vitro clone cultures by 1992. Since 1994, the EVIKA laboratory lost the possibility to multiply the protected varieties leading to a sudden decrease of the certified seed potato production area from 1300 ha to 76 ha in 1999 (Rosenberg, 2002).

As indicated in the Table 2, all official samples of ditylenchids in recent years in Estonia have been taken due to tasks not concerning potato rot- or stem- and bulb nematode monitoring.

The problem in Estonia was alleviated by starting to use in-vitro cultured basic material, informing state farm agronomists and seed potato producers of potential threats and by changing the varieties to more resistant or tolerant ones. In the middle of the 1990s, neither stem nor potato rot nematodes were considered among the 55 major crop pests and 50 diseases for the annual national prognosis survey (Taim & Soobik, 1994). Re-appearance of the problems more than ten years later, indicates that Estonian soils may have been carrying low levels of infestation ever since it was first discovered in the middle of the 20th century. Collapse of the Soviet seed potato propagation and national surveillance system in 1990s may have been another serious setback from the potato health point of view.

Table 2. Results of national surveillance sampling (reason indicated) and findings of *Ditylenchus destructor* (DDE) and *D. dipsaci* (DDI)

Year	Seed potato tuber analyses			Ware potato bacterial disease monitoring		Tuber analyses on producer's request	
	No of samples	Positive DDE	Positive DDI	No of samples	Positive DDE	No of samples	Positive DDE
2003	97	0	0		6		0
2004	80	0	0		0		0
2005	151	2	0		2		0
2006	195	0	0	357	3		0
2007	156	0	1		1	3	1
2008	176	2		229	9		
2009		1		210	16		
2010				252	5		

Data source: Estonian Agricultural Board, annual plant health surveillance reports, www.pma.agri.ee (last accessed 14. 06. 2011).

Some countries have recommended soil fumigation to destroy harmful nematofauna. Soil solarization may be considered ineffective due to lack of sun radiation intensity in our latitude. In Estonia, karbation has been recommended as a soil disinfectant back in the 1960– 1970s (Krall, 1965; Kikas, 1969). Reluctance of plant producers to adopt this as a common practice due to its toxicity to plants has helped to preserve natural biological diversity in soils (Krall, 2000). In the lack of effective control measures against potato rot nematode, agrotechnological methods have been suggested.

2.2. Factors affecting invertebrate overwintering

2.2.1. Negative temperature extremes in pest control (VI)

The geographic range of pest populations are limited mainly by the availability of host plants and the suitability of the climatic conditions. Low temperatures affect critically biological entities and play a predominant role in establishing the distribution limits of organisms (Renault *et al.,* 2002). In ectothermic organisms, the rates of biochemical processes decrease rapidly with dropping temperature. Insects and other organisms have evolved complex physiological and biochemical responses to cope with multiple environmental stressors (Holmstrup *et al., 2*002).

The extent to which pest managers can employ negative temperature extremes to protect the crop depends upon their ability to manipulate exposure of pests typically to naturally-occurring cold temperatures by removing insulation (Hoy, 1997). Snow is well known for its low thermal conductivity insulating the underlying soil, with a significant effect on physical and biological processes. Milner *et al.* (1992) have shown a successful attempt to manage Colorado potato beetle, *Leptinotarsa decemlineata* Say using an artificially mulched trap crop. In the 0– 15 cm soil strata, where most of the adult beetles overwintered, after the removal of mulch and snow cover, temperatures dropped from 0 to -11.7 °C, whereas in undisturbed plots, temperature remained close to 0 °C. Beetle survival was significantly higher in snow-covered, unmulched controls and mulched habitats (=26%) than in disturbed habitats (=7%) (Milner *et al., 1*992).

Even though some earlier work suggest that preventing snow covering the

ground has no significant effect on the relative abundance of soil nematodes (Sulkava & Huhta, 2003), others have demonstrated that freezing plays an important role both in soil physical properties and community composition, as reviewed by Henry (2007).

Early winter tilling or turning of the soil is a well-known crop protection practice in vegetable gardens, because it exposes over-wintering pests to winter conditions. This has been a recommended control strategy also for limiting root-nematode survival (Heinz & Goellner Mitchum, 2010). For North-West Russia, among other specific pest management measures, snow rolling to reduce soil temperature in the depth of tillering node against snow mould has been recommended in winter cereals (Nikolaev, 2009).

Weed suppression has been reported as one of the key factors to potato rot nematode control (Andersson, 1971). However, in modern farming, there are rising concerns about the effect of weed control regimes on biodiversity, as higher biodiversity usually confers greater ecosystem stability. Hence, rather than eliminating species, the task is to find methods to restore and maintain natural balance within the cropping system.

2.2.2. Cold tolerance in nematodes (V)

Within the limitations of restricted choice of nematicides available in Europe (none of which are currently registered in Estonia), nematode control efforts have to cope mostly with alternative strategies even in intensive agriculture. Because potato rot nematode is capable of remaining viable in soil (**II**), the presence of economically significant contamination of the field depends to a great extent on the level of overwintering survival.

Insect cold hardiness theory could not be directly applied to nematodes. Nematodes are essentially aquatic organisms and ice formation in the external water is likely to nucleate the body content freezing (Wharton, 2002). Nematodes, which survive low temperatures in their natural habitats, are cold tolerant (Wharton, 2002). The main model for studying nematode cold tolerance has been an Antarctic nematode *Panagrolaimus davidi* (Wharton, 2002, 2003), which exhibits atypical survival abilities when compared with other species of nematode (Smith *et al.,* 2008). It has been suggested, that most cold-tolerant nematodes are freezing-tol-

erant (Wharton, 2002). Freeze-avoiding strategy can be employed by a nematode, if it was free of surface water but not desiccated and supercool or in the presence of water supercool in an eggshell or a sheath. If they can survive anhydrobiotically, they would survive low temperatures without the risk of freezing.

Plant nematodes employ various cold tolerance strategies. The potato cyst nematode, *Globodera rostochiensis*, overwinters as second stage larvae (J2), within eggs contained within a cyst, which is formed of a body of the female nematode. Both, eggshell and the cyst act as barriers to ice nucleation (Wharton, 2002). The cysts endure desiccation and the enclosed larvae may survive anhydrobiotically (Perry, 1999).

The ability of some plant-parasitic nematodes to withstand dehydration for periods longer than the duration of the normal life cycle is often associated with a dispersal phase. In addition to behavioural, morphological and biochemical adaptations to dehydration, cyst- and root-knot nematodes endure long-term adverse conditions by egg diapause, which may be broken by a complex of cues, a.o. photoperiod and chilling (Wright & Perry, 2006). Hatched larvae of *G. rostochiensis* have been shown to suffer from external inoculative freezing and to die. Larvae within the eggs employ a freeze avoidance and/or protective dehydration strategy (Holmstrup & Westh, 1995). A pre-freeze acclimation effect was demonstrated in root-knot nematode *Meloidogyne hapla* larvae, the survival of which in polyethylene glycol or in soil was enhanced after acclimation (Forge & MacGuidwin, 1992).

Stem nematode cold survival (V)

The majority of recent nematode survival studies have been dedicated to *D. dipsaci* which unlike *D. destructor* is a fast-dehydration strategist (Womersley *et al.,* 1998). Several authors have observed survival of ditylenchids in field conditions. According to Ustinov and Linnik (1955), living *D. destructor* were discovered in the spring of 1954, before the planting of potato in fields used for potato growing the previous season. A related species, the bulb nematode *D. dipsaci*, survived after overwintering on the surface and at a depth of 10 – 50 cm in Gorky Oblast, Russia (Kir'yanova, 1951).

Nematodes, as aquatic organisms, are subjected to inoculative freezing when the water in their external environment freezes, unless they are able

to prevent it by the presence of protective structures such as an eggshell or sheath (Wharton, 1995). Without a resistant resting stage, the potato rot nematode overwinters in soil as adults or juveniles and may multiply by feeding on alternative weed hosts and on fungal mycelia. Its survival in soil is enhanced, for example, by the presence of unharvested potato tubers. In *D. dipsaci*, the most cold resistant stage has been found to be the anhydrobiotic fourth-stage juvenile (J4) (Bosher & McKeen, 1954). Thorne (1961) reported *D. destructor* overwintering in USA field soil as eggs and coiled adults. Ustinov and Tereshchenko (1959) suggested that the winter survival of potato rot nematode is facilitated by egg anhydrobiosis, which makes it capable of enduring low temperatures almost to the point where the soil freezes.

D. dipsaci has been reported to be freeze-susceptible but avoids freezing by supercooling with a mean supercooling point (SCP) of -21.7 ± 0.7 °C (Wharton *et al., 1*984). Stem nematodes remain mobile almost until their freezing point (Shubina, 1985). According to Ladygina (1957), the potato and onion Ditylenchids do not stop moving at 3– 5 °C, even though they become less active. In infested tubers, bulbs and soil they remain capable of invasion, reproduction and development even after being frozen to -18 to -28 °C for 2 days or -11 to -27 °C for 14 days. Makarevskaya (1983) reports *D. destructor* survival in plant tissues at subzero temperatures up to -2 °C and mortality at -4.5 °C and Ladygina (1957) states the lower lethal temperature of the species lies within the temperatures below -20 to -28 °C (Ladygina, 1957). Yet, there is no information on which life stages were used in the above-mentioned cold tolerance studies.

2.2.3. Overwintering strategies in invertebrates (III-V)

Research in invertebrate cold tolerance has mainly been focussed on insects or other arthropods (Wharton, 2002). Most biological processes and activities slow down at subzero temperatures or can even be stopped (Leather *et al., 1*993). The duration of survival is specific for each organism and may depend upon relative humidity, temperature acclimation or seasonal adaptations prior cold exposure, duration of exposure and naturally is highly dependent of the selected microhabitat (Renault *et al.,* 2002).

Organisms have developed various behavioural and physiological strategies to survive severe winter conditions (Lee, 1989, 1991; Block, 1991,

1996; Bale, 1996; Danks, 1996). In preparation for winter, they may stop feeding, seek a favourable microhabitat, empty their digestive system, undergo dehydration, and synthesize polyols and proteins to protect them from freezing (Zachariassen, 1985; Sømme, 1999). Desiccation could enhance survival by increasing osmolality of the haemolymph and concentrating cryoprotective substances (Zachariassen, 1985). Freeze-avoiding invertebrates prevent freezing at subzero temperatures by supercooling while freezing-tolerant ones survive intracellular ice formation (Lee, 1991).

Many invertebrates rely on supercooling up to a certain level of low temperature i.e., the supercooling point (SCP), at which their body fluids freeze. The cold hardiness of an insect species has been widely assessed by determining the SCP. However, this is not a reliable indicator for all species because, in some, mortality occurs at temperatures above or below their SCP (Bale, 1993; Carrillo *et al., 2*005). Thus, for example, SCP is an accurate predictor of cold hardiness for a ladybird, *Coccinella septempunctata* (Nedvěd *et al., 1*995) but not for a moth, *Mamestra configurata* (Turnock *et al.,* 1983). Some mortality may occur at temperatures above the SCP because of chill injuries (Lee, 1991). The SCPs are widely used as a comparative cold tolerance indicator in different physiological stages of an insect (Hodkova & Hodek, 2004).

Cold hardiness is a common strategy of organisms to survive cold periods and it is influenced by many environmental factors like food, temperature, and humidity (Sømme, 1999). In Northern regions with unstable winters, cold hardiness is an inevitable prerequisite for winter survival. Cold hardiness is defined as the ability to survive exposure for longer periods at low temperature. In many species, mortality increases with prolonged exposure to subzero temperatures or with decrease in temperature (Block, 1995; Sømme, 1999). The length of exposure has been considered the most important factor determining mortality because extremely low temperatures are usually softened by snow cover (Bale, 1991). Prolonged exposure at subzero temperatures may cause injury and mortality even if the insect does not freeze (Renault *et al.,* 2002).

Many insects, when exposed to cool temperatures for days or weeks in the autumn, become acclimated, and their cold hardiness increases aiding their survival during cold periods (Bale, 1989; Sømme, 1996; Renault *et al., 2*002). Rapid cold hardening response may protect against a non-

freezing injury known as cold-shock or direct-chilling injury which occurs at temperatures 5 °C or more above the SCP (Lee & Denlinger, 2010).

Small striped flea beetle – *Phyllotreta undulata* Kutchera, 1860

Flea beetles (Coleoptera: Chrysomelidae) inhabit a wide range of environments where cruciferous plants grow, including fields, gardens, and uncultivated areas. Their population densities vary widely between years (Hiiesaar *et al., 2*004). Flea beetles have been much studied (e.g., Burgess, 1977; Nielsen, 1977, 1996; Kinoshita *et al.,* 1979; Wylie, 1979; Lamb, 1983; Andersen *et al., 2*005) but there is little information on their cold hardiness.

Flea beetles are univoltine (insects with an obligate diapause, giving only one generation per year). They overwinter as adults, usually outside fields, in margins, in hedgerows, and beneath shrubs, although some find shelter within fields in leaf litter, in stubble, or in grassy areas (Andersen *et al., 2*005; Ulmer & Dosdall, 2006). Physical conditions at their overwintering sites may fluctuate daily, seasonally, and between years. As they do not burrow deep into the soil, their survival over winter depends on winter temperatures and the depth of the snow layer.

Pollen beetle – *Meligethes aeneus* Fabricius, 1775

Meligethes aeneus (Coleoptera: Nitidulidae) is a univoltine species which causes serious damage to cruciferous crops and many other cruciferous plant species (Williams, 2010). Before hibernating, most adults migrate to forests, brush, or hedges where they are more insulated from the temperature extremes of open fields. Adults spend the winter in obligate diapause which is broken only after a certain cold period, usually before the end of January (Marczali & Nádasy, 2006).

The life history of *M. aeneus* has been extensively investigated (Williams, 2010). Studies include mortality factors of the larvae and adults (Büchi & Nuss, 2000; Büchi, 2002; Hokkanen, 2008) and data about hibernation of beetles under field conditions in both northern (Hokkanen, 1993; Lehrman, 2007) and southern regions of Europe (Marczali & Nádasy, 2006). According to Hokkanen (1993), mortality of hibernating pollen beetles in Finland may reach 85–98%. Lehrman (2007) found only 2.4% winter survival in 2006 in Sweden. Adults, embryos and larvae may all encounter low temperatures, because, in northern regions, temperatures around zero with night frosts are common during the growing season.

3. AIMS OF THE STUDY

Domestic potatoes as staple food are of strategic importance for quality food provision. Potatoes belong in the range of primary cash crops for many plant producers and households in Nordic temperate regions. Recent re-appearance of problems due to potato rot nematode comprises a serious food security breach, influences the competitiveness of potato growers and consequently may damage social welfare in rural communities.

1. We assume that distribution and spread of *Ditylenchus destructor* in potato growing areas is favoured by several anthropogenic factors: trade volume and market demand, absence of legislative measures and targeted research programs, cultivation practices and availability of control options. A database survey **(I)** and literature review **(II)** were conducted to summarize the existing international and national experience with the pest within the context of current agricultural trends.

2. Distribution of the potato rot nematode is affected also by various ecological factors. Winter conditions delimit the spread and population densities of most economically significant pests in Nordic Temperate areas. Yet, there is no knowledge on how the potato rot nematode has been able to tolerate the overwintering stress, neither are there methods for estimating their cold hardiness. Firstly, the techniques employed for flea beetles (*Phyllotreta undulata*) **(III)** and pollen beetles (*Meligethes aeneus*) **(IV)** were tested to develop the advanced methodology for nematode cold hardiness studies. Secondly, since the potato rot nematode overwinters in all life stages, possibly both in free-living as well as endoparasitic state, the cold tolerance of hatched juveniles and adults (post-eclosion stages) of *D. destructor* in various aqueous environments, as well as in plant tissues **(V)** was examined.

3. Although, major pathways for the potato rot nematode are ware and seed potatoes, there are indications that Estonian soils may have been carrying low level of infestation since the 1950-s. Therefore, methods alternative to seed potato health requirement, extensive chemical treatment and standard crop rotation practices need to be investigated. In order to test the hypothesis that natural winter frosts may be employed to reduce pest populations and to estimate the influence of soil temperature manipulations on the potato rot nematode winter survival **(VI)** the effect of snowpack densification and snow removal on the soil temperature were studied.

4. MATERIALS AND METHODS

4.1. Data survey: The effect of trade on distribution of *Ditylenchus destructor* (I)

Data was retrieved from the «EPPO Reporting Services», which are regularly published by the European and Mediterranean Plant Protection Organization (EPPO), where notifications of noncompliance (detection of regulated pests) are reported by the member countries. The analysis considered all the pest interceptions having occurred in the eleven years from 2000 to 2010. Since EPPO has expanded to Northern Africa and Western and Central Asia, some species indigenous to Europe are likely to be of regulatory concerns in those areas. According to the availability of data, the reports of a total of 28 countries were thus considered.

According to the United Nations geoscheme (United Nations Statistics Division, 2011), the countries were separated into following regions:

- Western Europe: Austria, Belgium, France, Germany, the Netherlands, Switzerland,
- Northern Europe: Denmark, Estonia, Finland, Ireland, Latvia, Lithuania, Norway, Sweden and the United Kingdom
- Southern Europe: Greece, Slovenia and Spain
- Eastern Europe: Bulgaria, Czech Republic, Hungary, Poland and Russia
- Northern Africa: Algeria and Tunisia
- Western Asia: Cyprus, Israel and Turkey

The following variables were surveyed:

1) Relative importance of different nematode families and major species intercepted in the international plant trade,
2) Relative importance of associated commodities and
3) Origin classified into the regions according to the categories of the United Nations geoscheme.

The survey data on most important plant parasitic nematodes was collated in order to calculate the relative indices on establishment success. Comparison of the indices by the nematode groups was performed by Chi-square test.

4.2. Laboratory experiments: The effect of media and cold on invertebrate survival (III-V)

4.2.1. Subjects

The beetles were field-collected from crops growing in the vicinity of Tartu, Estonia (58°23'N, 26°41'E) **(III, IV)**. Two generations of *Phyllotreta undulata* were collected from white cabbage **(III)**. Overwintered flea beetles collected in early June 2007 were used, whereas pre-winter beetles were collected in August–September 2007.

The experiments with *Meligethes aeneus* were performed in 2008, 2009, and 2010 **(IV)**. Overwintered pollen beetles were gathered from dandelions and winter oilseed rape buds and flowers in May and June. New generation beetles were collected in August and September from flowering milkweed, Canada thistle and nasturtium. As *M. aeneus* is difficult to find in its overwintering sites, some beetles were placed in 1-l glass jars, filled to one-third with lightly moistened peat covered with crinkled paper where the beetles could hide. The jars were held at +2 to +4 °C until use.

Nematode-infested potato tubers were collected from Viljandi county, Estonia (58°29'N, 25°36'E) in January 2010 **(V)**. The stock material was stored at +4 °C for the duration of the experimental series.

4.2.2. Laboratory equipment and measurements

The technology was designed, installed and adapted for insect physiological research by the scientists of the laboratory of experimental entomology in the Plant Protection Department of the Estonian University of Life Sciences. For the purposes of the current thesis work, the existing equipment and methods were modified to satisfy the limitations of nematological cold tolerance experiments.

SCP of *Phyllotreta undulata* and *Meligethes aeneus* (III, IV)
Supercooling points were measured using a chromel-alumel thermocouples-thermometer (RS-232, Data logger Thermometer; TES Electrical Electronic, Taipei, Taiwan). Low temperatures (down to –30 °C) were attained by deep-freezer Haier HF-103. Prior to supercooling, beetles were cooled to a low temperature (0 to –2 °C) to immobilise them. Each

was then fixed individually to the top of the thermocouple with a thin layer of vaseline, and sealed in a plastic tube. The tubes were placed in a cotton-lined container and transferred into the freezing chamber at -30 °C. As cooling rate can affect the SCP, we chose a standard rate of 1 °C min^{-1} (Salt, 1966; Merivee, 1978; Cannon, 1983). The temperature at which latent heat was released during freezing was taken as the SCP of the individual. Since gender does not affect SCP of *P. undulata* (Muljar *et al., 2*007) or *M. aeneus* (**IV)**, mixed sex beetles were used in all experiments. The number of beetles used per experiment varied. To obtain field-collected *M. aeneus* beetles with empty guts, they were held at room temperature for 24 hours with no access to food before starting measurements.

Short exposure to sub-zero temperatures of non-acclimated and acclimated overwintered *P. undulata* (III)

Overwintered beetles kept at 0 °C for 7 days in the laboratory (acclimated) were compared with overwintered beetles used directly after collection from the field (non-acclimated). Groups of 20–56 acclimated or non-acclimated beetles were each enclosed in a 25-ml plastic tube and cooled in the deep-freeze to a temperature of -4, -6, -8, -10, -12, -14, -16, or -18 °C, at a cooling rate of 1 °C min^{-1}. They were kept at these temperatures for 1 min and then re-warmed to room temperature at the same rate as they had been cooled. The cooling profile was monitored with a thermocouple placed inside each tube with the beetles. Mortality was determined after 2 hours; any beetle not moving or responding to tactile stimuli was considered dead. The experiment was replicated four times. To determine whether the SCP of the beetles is a reliable indicator of their cold tolerance we compared it with the LT50 of non-acclimated and acclimated beetles.

Long exposure to sub-zero temperatures and the cold intensity tolerance of *M. aeneus* (IV)

The effect of long-term exposure to constant low temperatures on mortality of beetles ('quantity factor'), and the ability to withstand extreme low temperatures ('intensity factor') (Payne, 1926, as cited in Renault *et al.,* 2002) was assessed. The experiments were conducted in four distinct cohorts: after emergence of beetles from overwintering places (early June), after emergence of a new generation beetles from the soil (early August), before the beetles left for their overwintering sites (September), when the beetles were in deep diapause (mid January; quantity factor), or

after termination of diapause (late February; intensity factor).

The quantity factor tolerance was determined at constant -7 °C. This temperature was selected because it was higher than the beetle's mean SCP and a temperature the pollen beetles were likely to encounter in their overwintering sites under field conditions. Batches of beetles were wrapped in glass vials lined with filter paper and transferred into the thermostat for various periods of time: 24, 48, 72, 96, 120, 144, and 312 h. One batch per treatment was removed at daily intervals. In the intensity factor experiment, the beetles were exposed for a constant period of time (24 h) to various subzero temperatures: -7, -9, -11, -13, -15, and -17 °C.

As in the SCP and short exposure experiments, the beetles were cooled at a rate of 1 °C min^{-1}, and re-warmed to room temperature at the same rate. The temperature fluctuation inside the vials did not exceed 0.5 °C. Constant cooling and warming rates were achieved using a liquid thermostat (Ministat 230w-2; Huber, Offenburg, Germany; -33 to 200 °C). Following exposure, the beetles were kept in Petri dishes at room temperature. Low temperature effect was scored twice: development of chill (cryo) injuries and mortality was assessed 2 h after transfer of beetles from cold to room temperature. Based on the method described by Košťal *et al.* (2007), the beetles were considered fit if they were active and able to move in a normal manner. Beetles were considered injured if they fell over and could move only short distances. Beetles not responding to tactile stimulation were considered dead. The second assessment was made after 24 h (sometimes 48 h) by which time the beetles had either recovered from their injuries or were obviously dead.

Survival of *D. destructor* in various media (V)

In order to limit overestimation of mortality, several media were tested for simulating nematode survival environments. Hand-picked adults were placed into 1.5 ml Eppendorf tubes containing either 200 μl water, nematode buffer M9 (De Ley & Mundo-Ocampo, 2004) or gravel moistened with water. A preliminary cooling trial had resulted in the choice of the buffer M9 out of a group of solutions with the same specific gravity, which are used in standard nematological practices, since it remains liquid at high subzero temperatures. Additionally, samples of 0.268 ± 0.025 g of infested potato tissue (standard sample) were taken by a cork borer size 1 (internal diam. 3.5 mm). In total, eight replicates were made with water, five with gravel and potato and 11 with M9 buffer. The number

of replicates in water and buffer was increased in order to get more reliable results. The samples were cooled and reheated at 1 °C min^{-1}. After 24 h cold exposure at -5±1 °C in the liquid thermostat, nematodes were removed from the thermostat and kept at +4 °C for 24 h to recover before estimation of survival. Mortality was determined by counting the individuals that did not respond to mechanical stimulation by a bristle.

Cold intensity tolerance of *D. destructor* (V)
For evaluation of nematode cold tolerance, a novel thermocouple method was developed on the basis of the technique used with *M. aeneus*. Standard samples of infested potato tissues were contacted to a thermocouple and transferred into 1.0 ml Eppendorf tubes to monitor the temperature fluctuation. The Eppendorf tubes were placed in test tubes into the liquid thermostat and exposed to temperatures of -5, -8, -18 or -30 °C for 24 h. In order to assess the effect of cold exposure on the population, nematodes were counted and the survival of different post-eclosion stages was assessed after a 24 h recovery period. The counts of younger juvenile stages, second- (J2) and third-stage (J3), were combined. The results were compared to that of control samples kept at room temperature (+22 °C) for the same period of time. There were five replicates at each temperature.

4.2.3. Data acquisition and statistics

Seasonal changes in SCP of *M. aeneus* were analysed with Tukey's honestly significant difference (HSD) test for unequal sample sizes. Differences in the survival rate of *M. aeneus* between seasonal groups depending on exposure time or temperature were compared by two-way ANOVA. In addition, lethal time to reach 50% mortality of beetles (Ltime50) after exposure to constant -7 °C, and lethal temperature at which 50% of beetles died (LT50) when exposed for constant 24 h were calculated by probit analyses (Finney, 1962).

Temperature-dependent mortality of *P. undulata* after 1 min exposure to different low temperatures was analyzed with one-way ANOVA. LT50 was calculated by Probit analysis.

The probit models with SAS 9.1 (SAS Institute, Cary, NC, USA) procedures PROBIT and GLIMMIX were fitted to estimate and test the effects of media, temperature and age on the survival of *D. destructor*.

The discrete effects of media in the first experiment and of age and temperature in the second experiment were compared using suitably defined contrasts. Additionally, the continuous changes in mortality were modelled and the lethal temperatures for 50 and 90% mortality of nematodes were estimated considering the temperature as a continuous factor and applying the probit-regression corrected for control mortality at 22 °C using Abbott's formula. A p value < 0.05 was considered statistically significant.

All analyses were performed with SAS/STAT version 9.1 (SAS Institute, Cary, NC, USA).

4.3. Field experiment: The effect of snow treatments on soil temperature (VI)

4.3.1. Experimental setup

The experiment was conducted at the Tõnisson Experimental Garden, Estonian University of Life Sciences, at Eerika near Tartu, Estonia (58°21'N, 26°40'E) on sandy loam Stagnic Luvisol in WRB (FAO, 1998) classification (Reintam *et al.,* 2008) from 13 January 13 until 12 April 2011. The test plots were located at the centre of an open field with winter rye as a cover crop (cabbage as previous culture) in a radial arrangement in three replications. Due to ethical and licensing considerations, the experimental garden was not contaminated with infective nematodes, but the temperature records were planned to be compared with the results of the laboratory experiment.

After the demarcation of the plots and digging out the bare ground under the natural snowpack of 52 cm, the data loggers (LogTag Model: TRIX-8 Temp. Logger, Micro *DAQ*.com, China), isolated from the soil humidity, were buried in 5 cm soil as it has been demonstrated that *Ditylenchus dipsaci,* a species related to *D. destructor*, is most abundant in crown soil of the host plant (Simmons *et al., 2*008). The snow cover was carefully restored in control and snow densification treatments.

To increase density and reduce the isolation capacity of the snow, 50 cm diameter plots were trampled down by boots into a flat solid base, which measured 17– 21 cm in depth. To test the effect of reduced snowpack

thickness, snow was completely removed on 50 cm diameter plots. Even when the snowpack of the control plots was compacted during the onset of the experiment because of unavoidable disturbances while the data loggers were set in their places, the original snow cover thickness was quickly restored by snowfall and wind, which resulted in 60– 63 cm deep snowpack by 26 January, when the natural undisturbed snow layer measured 63 cm.

To keep the fresh snow accumulation on treated plots at less than 10 cm level by the arrival of a cold front, the snow treatments were carried out on 19, 24 and 26 January and 14 February. The arrival of the cold front was recorded on 12 February, when the mean daily air temperatures fell below -10 °C (min. -17. °C) and it lasted until 25 February when the mean temperature was -13.2 °C (min. -21.5 °C).

4.3.2. Measurements and statistics

Measurements were programmed to be recorded automatically every 30 minutes. The data loggers were excavated and the measurements retrieved in spring after the snow melt and daily average temperature rose above 0.5 °C. The effect of snow manipulations was compared by repeated measures ANOVA, for the period after the last snow treatment on 14 February until the passing of the cold front. The temperature records were compared with the results of an earlier laboratory experiment.

5. RESULTS

5.1. *Ditylenchus destructor* in international trade (I)

Our analysis of the relative importance of nematodes in the international plant trade reported by the member countries of European and Mediterranean Plant Protection Organization (EPPO) from 2000– 2010 shows that potato rot nematodes were present in about 3% of 221 potato lots intercepted with nematode contamination whereas 90% of interceptions were caused by potato cyst nematodes. Nevertheless, *D. destructor* was among the ten best recognised phytonematodes in the international plant trade, although it has been identified to species level only in 8 cases while *Globodera rostochiensis* has been reported in 69 and *Bursaphelenchus xylophilus* in 60 cases for the same period. Nematode families (sub-families) intercepted in international consignments during phytosanitary checks are presented in Figure 4.

Of the 1088 nematode interceptions that occurred between 2000 and 2010, the vast majority concerned nematodes related to bonsai or aquatic

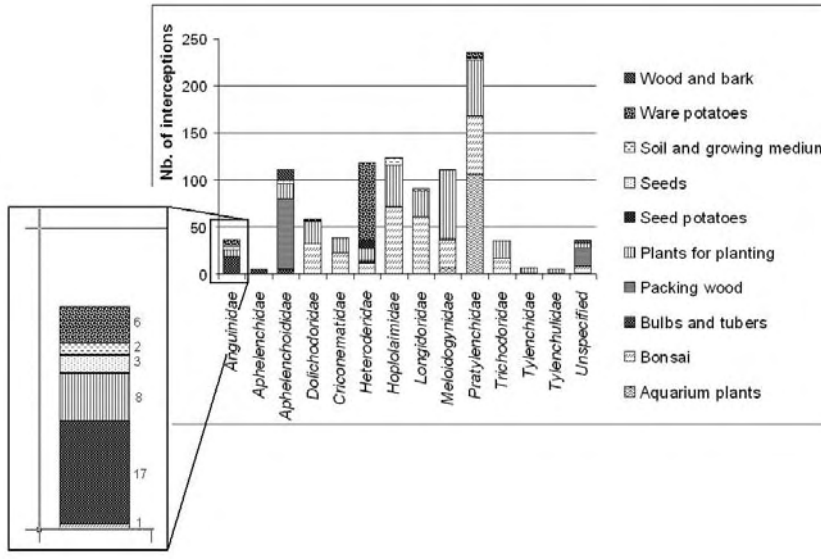

Figure 4. Nematode groups associated with major commodities intercepted by EPPO member countries from 2000– 2010. Genus *Ditylenchus* belong in the family Anguinidae.

plants. The origin of alien nematodes differed significantly depending on the associated commodity (Chi square test; $p = 0.000$). The nematode species arriving with bonsais and wood packaging material originated predominantly from Asia (96.7 and 76.9%, respectively) whereas those arriving with timber and logs came from Russia (47.9%) but also through intra-European exchanges. In addition, the diversity of nematodes carried by bonsais was significantly higher than that of the nematofauna carried by potato or timber products (Chi square test; $p < 0.01$).

The majority of the ware potato consignments in our survey originated from Europe (66 interceptions). To a lesser extent, other continents contributed: Western Asia with 25 consignments and Northern Africa with 3 consignments. Potato rot nematode was found by Bulgaria in 2 consignments from Turkey and by Lithuania in 4 consignments imported from Eastern European countries (Belarus, Moldova, Poland, Hungary).

Altogether, 879 reports were reviewed with the details of 1088 nematodes, 318 of which concerned nematode infested bonsais, 312 plants for planting, 114 aquarium plants, 113 wooden packaging materials, 97 ware potatoes, 28 bulbs (tubers) of ornamentals or vegetables excluding potatoes, 26 raw wood, 16 soil or growing medium, 7 seed potato, 6 seeds and 6 other materials.

Of the 1181 consignments intercepted by the EPPO member countries between 2000 and 2010, 678 were reported by the Western European countries, including 245 with bonsais, 186 of plants for planting and 167 of aquarium plants. In Northern Europe, 362 consignments were found infested with the major risk commodities being ware potatoes, wooden packaging material and plants for planting (138, 115 and 44 interceptions, respectively). In this group, bonsais were intercepted due to the plant nematodes only by the United Kingdom, whereas aquarium plants were intercepted by Denmark. The major nematode concerns for Eastern and Southern European countries were associated with ware potatoes (66 interceptions of total 111 by the former) and raw timber (5 reports of total 9 by the latter). Western Asia contributed with 16 interception reports and Northern Africa with 5 reports.

Intercepted seed potato was exclusively of Western European origin. Only potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) were found in seed potatoes.

5.2. Media and cold as factors influencing invertebrate survival

5.2.1. Optimization of research methods (III, IV)

A series of cold hardiness and supercooling experiments with *Phyllotreta undulata* and *Meligethes aeneus* were conducted to find methods suitable for application in *D. destructor* cold tolerance research as shown in Table 3. As a result, overwintered mixed culture embedded in natural host plant with J2-4 and adults was chosen to be targeted in testing the mortality after 24 h exposure to constant extreme low temperatures.

Table 3. Immediate availability of materials and applicability of the insect cold tolerance research methods for nematode studies in the laboratory of experimental entomology of the Estonian University of Life Sciences.

Age group	Tests	Ref	Availability for application with nematodes	Risks
Eggs	Supercooling point	IV	Special extraction methods and thermoelectric microscope cold stage required.	External ice nucleation
	24 h exposure to extreme low temperatures	IV	Special extraction methods required	Manipulation stress
Larvae	Supercooling point	IV	Thermoelectric microscope cold stage required	External ice nucleation
Overwintered adults	Supercooling point	III, IV	Thermoelectric microscope cold stage required	External ice nucleation
	Short exposure to constant cold temp.	III	Available	
	24 h exposure to extreme low temperature	IV	Available	
	Long exposure to constant low temperature	IV	Available	
	Effect of food	III	Possibly available	
	Starvation	III	Possibly available	
	Acclimation	III, IV	Possibly available	
Young adults	24 h exposure to in extreme low temperatures	IV	Permanent nematode culture required	Contamination
	Long exposure to constant low temperatures	IV	Permanent nematode culture required	Contamination

(Table 3. Continued)

Age group	Tests	Ref	Availability for application with nematodes	Risks
Pre-winter adults	24 h exposure to extreme low temperatures	IV	Permanent nematode culture required	Contamination
	Long exposure to constant low temperatures	IV	Permanent nematode culture required	Contamination
	Effect of acclimation	IV	Permanent nematode culture required	Contamination
Winter adults	24 h exposure to extreme low temperatures	IV	Possibly available	
	Long exposure to constant low temperatures	IV	Possibly available	

5.2.2. The effect of media (V)

Mortality rates of *D. destructor* in different media ranged from 31 to 97% (Table 4). Most favorable for low temperature survival (and statistically not different, $F_{1,25} = 0.03$, $p = 0.86$) were potato and M9 buffer. The mortality in water and gravel was significantly higher than in potato and M9 buffer ($F_{1,25} = 145.90$, $p < 0.001$), but did not differ mutually ($F_{1,25} = 0.96$, $p = 0.34$).

Table 4. Effect of the media on mortality of *Ditylenchus destructor* post-eclosion stages after 24 h exposure to -5 ±1 °C.

	Water	Gravel	Potato	M9 buffer
No. replications	8	5	5	11
Range total no. per sample (min/max)	10/30	10/10	86/288	10/40
Range no. dead per sample (min/max)	9/30	7/10	30/70	2/10
Total no. dead/total no.	136/140	47/50	275/867	85/273
Total mortality ±SE (%)	97.1±1.4 a	94.0±3.4 a	31.7±1.6 b	31.1±2.8 b

Mortalities followed by different letters are statistically significantly different ($p < 0.05$).

5.2.3. The effect of subzero temperatures

***Ditylenchus destructor* (V)**

Mortality of the different stages of the potato rot nematode exposed to subzero thermal regimes ranged from 32 to 100% (Table 5). There were no statistically significant differences between age groups at 22 °C ($p > 0.05$). Cold tolerance of adults and J4 at -8 °C was significantly lower than that of younger juveniles ($F_{1,12} = 77.19$ and $F_{1,12} = 34.75$, respectively, $p < 0.001$). Also the difference between J4 and adults was statistically significant at -8 °C ($F_{1,12} = 24.66$, $p < 0.001$). At -18 °C there were no significant differences in mortality between adults and J4 ($F_{1,12} = 0.00$, $p = 0.98$), but the cold tolerance of J2 and J3 was still significantly higher ($F_{1,12} = 18.80$, $p < 0.001$). Studying the speed of changes in cold tolerance caused by the decrease of temperature, it appears that adults are the most sensitive and J2 and J3 the most resistant. The mortality of adults increased rapidly and was statistically significant only between temperatures -5 °C and -8 °C ($F_{1,28} = 162.21$, $p < 0.001$), whilst the mortality of juveniles changed more slowly, showing statistically significant changes down to -18 °C for J4 and -30 °C for J2 and J3 (Table 5). The same differences are visualized in Figure 5 where the mortality is modelled on a continuous scale. The variability in mortality is greater among younger nematodes than in adults, which react similarly to the temperature changes.

Table 5. Mortality of post-eclosion stages of *Ditylenchus destructor* after 24 h exposure to various temperatures.

Age group	No. dead/total no. (mortality ± SE, %)				
	22 °C	-5 °C	-8 °C	-18 °C	-30 °C
J2 + 3	15/50 (30.0±6.5) A	85/265 (32.1±2.9) a,A	821/1091 (75.3±1.3) a,B	237/268 (88.4±2.0) a,C	88/90 (97.8 ±1.6) D
J4	11/40 (27.5±7.2) A	78/177 (44.1±3.7) b,A	430/488 (88.1±1.5) b,B	242/245 (98.8±0.7) b,C	29/29 (100.0 ±0.0) C
Adult	7/28 (25.0±8.3) A	53/142 (37.3±4.1) ab,A	358/367 (97.5±0.8) c,B	179/179 (100.0±0.0) b,B	11/11 (100.0 ±0.0) B

Mortalities followed by different lowercase letters within a column and different capital letters within a row are statistically significantly different ($p < 0.05$).

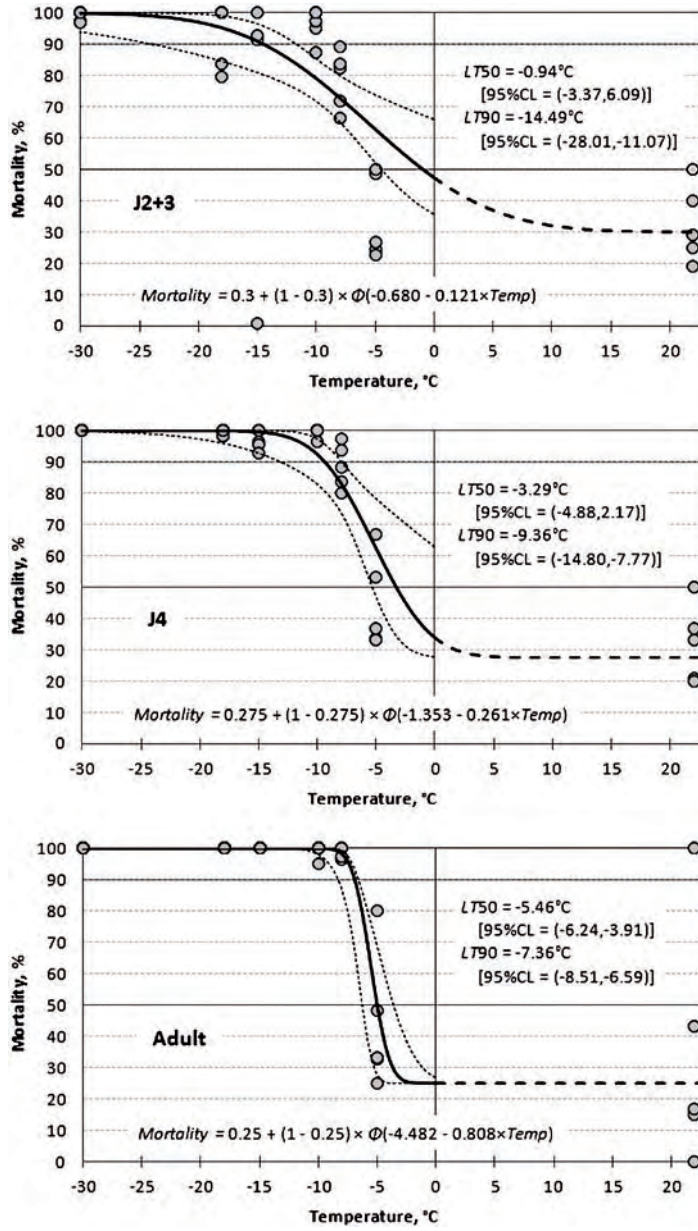

Figure 5. Cold tolerance of post-eclosion stages (adults, fourth-stage juveniles J4 and combined second- and third-stage juveniles J2+3) of *Ditylenchus destructor* after 24 h exposure to temperature regimes from -5 to -30 °C. Circles mark the mortality of different replicates, solid line (dashed line for positive temperatures) corresponds to the probit model corrected for control mortality at 22 °C using Abbott's formula and dotted lines are the 95% confidence limits of prediction.

The highest lethal temperature for 90% of population was exhibited by adults (-7.4 °C) when compared with J4 and younger juveniles (-9.4 °C and -14.5 °C, respectively). Adults exhibited 100% mortality at temperatures below -15 °C. Although external ice formation affects all life stages, a few J2 were able to survive temperatures as low as -30 °C.

***Phyllotreta undulata* (III)**

The effect of sub-zero temperatures was measured on SCP and mortality of non-acclimated and acclimated overwintered flea beetles with and without prior acclimation at 0 °C for 7 days. There was no mortality in either group at -4 °C and, in both groups survival was high at -6 °C (Fig. 1. **III**). Mortality increased gradually with decrease in exposure temperature (one-way ANOVA: $F_{6,21}$ = 149.247 and 396.069, both $p < 0.001$ in non-acclimated and acclimated beetles, respectively). All non-acclimated beetles died at -18 °C, a few acclimated beetles (2.4%) survived this temperature. Acclimation affected survival at most of the sub-zero temperatures tested. There were significant differences in mortality between non-acclimated and acclimated beetles at temperatures from -6 to -16 °C (Table 4. **III)**. Non-acclimated beetles died at temperatures 3 °C higher than their SCPs. The proportional difference between mean SCPs and LT50 remained the same in both groups, whether they were acclimated or non-acclimated to low temperature.

***Meligethes aeneus* (IV)**

The survival of *M. aeneus* beetles from various seasonal groups following 24 or 48 h after the end of cooling was estimated (Fig 6. **IV)**. Probit analysis data, with LT50 after exposure to constant 24 h period, are shown in Table 1, **V**. At -9 °C, chill injuries and mortality of summer beetles developed more or less equally; in the autumn group, the proportion of beetles with cold injuries was greater than the proportion of beetles that died; in overwintered beetles the reverse occurred. Temperatures of -11 and -13 °C caused mortality of most summer and overwintering beetles, few showed cold injuries; in the autumn group the proportion of beetles with cold injuries dominated overwhelmingly. After exposure to -15 °C, only a few beetles showed chill injuries, most of them were dead. Two-way ANOVA shows that season ($F_{3,59}$ = 117.15; p <0.0001) and temperature ($F_{5,59}$ = 3569,74; p <0.0001) had a significant effect on survival but that survival varied among seasonal groups (interaction: $F_{15,59}$ = 67.79; p <0.0001). Most vulnerable to cold exposure were overwintered reproductively active beetles in early June, the temperature of -9 °C was lethal

for most of them. Most resistant were beetles in late September after termination of feeding, when 70% of them survived exposure to -9.0 °C. Survival of the August group was much greater than in June but lower than in September. At the end of February, after termination of diapause, the beetles started to lose their cold tolerance as significantly fewer individuals survived exposure to -9 °C than in September (Fig. 6, **IV)**. When the temperature dropped to -11.0 °C, survival decreased substantially. None of the spring beetles survived this temperature; there were no significant differences among summer, autumn, or winter beetles with about 10% survival. Few autumnal and overwintered beetles survived exposure to -13 °C; only single autumnal beetles survived a temperature of -15 °C and all died at -17 °C. LT50 in *M. aeneus* showed a significant difference only with overwintered spring beetles; there were no significant differences among the other seasonal groups because the 95% fiducial limits overlap (Table 1, **IV)**. A temperature of ca -9 °C was the valid survival limit for beetles of the new generation after 24 h exposure.

5.3. Snow treatment for reduction of soil temperatures (VI)

Soil temperature readings as well as the mean air temperatures and daily precipitation are presented in Figure 6**.** There was a statistically significant difference in mean soil temperatures between the treatments ($F_{4, 142}$ = 498.7; p <0.001), with the significant interaction of date and temperature ($F_{44, 142}$ = 1.49; p = 0.04). Fisher LSD analysis revealed that snow removal treatment was significantly different from the other manipulations in all the compared measurement points, while the differences between the control and the soil densification treatment appeared only after 19 February. While the minimal subzero temperatures during the passage of the cold front in control did not exceed -0.8 °C, the snow densification and snow removal manipulations resulted in minimum temperatures of -2.1 °C and -10.3 °C, respectively. This indicates that LT50 of the younger juvenile stages of potato rot nematodes may be obtained by snow densification but more laborious snow removal may result in soil temperature drop to the level LT90 of older juveniles and adults (**V**), even though the lower lethal temperature for adults (-15 °C) was not achieved.

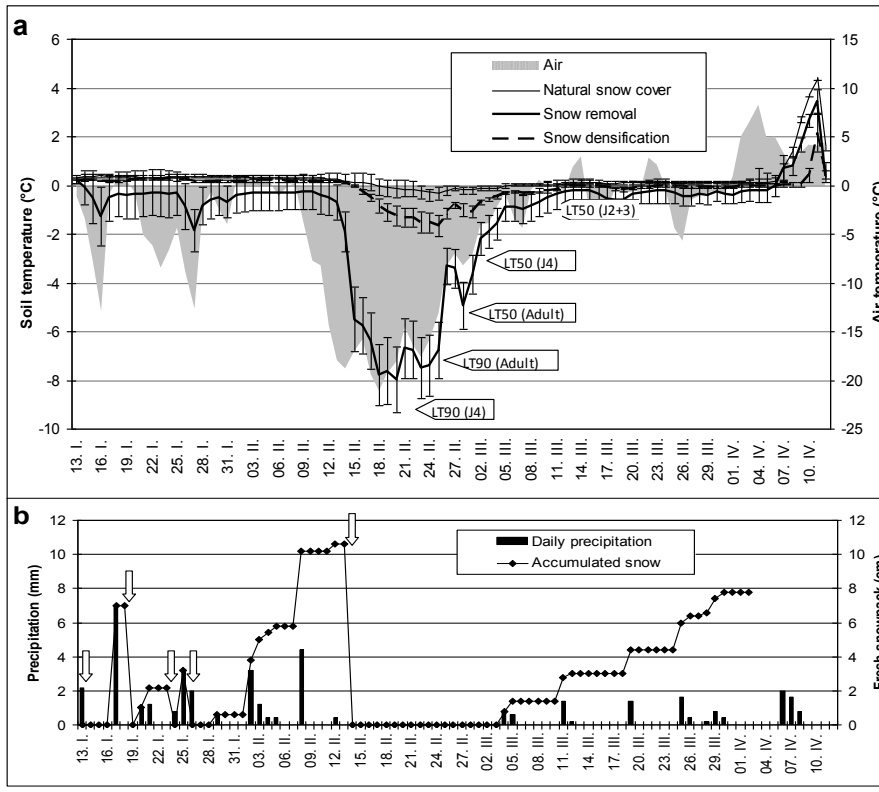

Figure 6. (a) The effect of snow treatments on mean (±SD) soil temperatures at 5 cm depth at the Tõnisson Experimental Garden, Estonian University of Life Sciences, Tartu, Estonia (58°21'N, 26°40'E). Block arrows indicate lethal temperatures for 50% (LT50) and 90% mortality (LT90) of post-eclosion stages (adults, fourth-stage juveniles J4 and combined second- and third-stage juveniles J2+3) of potato rot nematode after 24 h exposure to subzero temperatures (according to **V**).

(b) Mean daily precipitation (according to the Laboratory of Environmental Physics, http://meteo.physic.ut.ee) and accumulated snow cover thickness (calculated roughly by the rule: 1mm precipitation = 1cm snow). Block arrows denote the dates of snow treatment.

6. DISCUSSION

6.1. The effect of trade on distribution of *Ditylenchus destructor* (I, II)

Analysis of the interception data showed clearly that the origin of consignments plays a critical role in nematode incidence rate. Our survey results support the theory by Blumler (2006), indicating that the number of introduced species present correlates with the number of different early trade routes, frequency of intentional introductions, trade volume (particularly of unprocessed agricultural products) and the size of the region receiving the introductions.

According to the EUROSTAT database, yearly ware potato trade in Europe from 2000 to 2010 totalled over 900 to 1600 billion Euros in value. A considerably smaller volume is imported from Western Asia or Northern Africa, with the same indicator ranging roughly between 50 and 100 billion Euros annually. Ware potato infestation rate with all nematodes detected by our survey was shown to be larger in consignments originating from Europe (70%) in comparison to third countries (30%). These data are roughly comparable to *D. destructor* findings. Recognizing the fact that international potato trade with third countries averages only 10% of the intra-European trade values, we may conclude that the import material is of significantly higher pest incidence and therefore higher phytosanitary risk.

The correlation between trade volume and the number of invasive species incursions has been reported by many authors (Levine & D'Antonio, 2003; Blumler, 2006; Svilponis *et al.,* 2010). In the case of weeds, Forcella and Wood (1984) envisaged a geographical rule that invasion success correlates with the size of a species' natural distribution. It has been suggested that the biological traits enabling some species to spread across their native territory also facilitate their invasion of new areas (Roy *et al.,* 1991). Due to the unknown history in the case of potato rot nematode, the foreign nature of the species in Europe can not be confirmed and it should be categorized as cryptogenic. Hence, its invasion success cannot be estimated without respective population genetic evaluations.

Comparison of the distribution maps (Fig. 1, 2 and 3) shows the limi-

tations of information for establishing the present range of the species. Apart from trade-policy, reasons may be the absence of recent findings at the regional level or the lack of nematology experts.

The ecological principle of Competitive Exclusion (Hardin, 1960, Speight *et al., 2*008) may help explain the variation in the presence of *D. destrucor* in Europe. Evidently, homogeneous habitats with relatively few available niches support fewer species than heterogeneous ones, since the species-rich habitat allows for more co-existence by enabling them to partition resources within the habitat and thus avoid inter-specific competition. Mosaics of mixed semi-natural habitats were characteristic of Eastern European agricultural landscapes, whereas human impact has been more disruptive in rural areas of Central and Western Europe. Such habitat mosaics are also considered to reduce the probability of extinction of rare species.

International trade with seed and ware potato has been extremely restricted by legislative measures. Ever since the first plant protection laws were passed, potato quarantine has been well addressed (Ebbels 2003). The European Union has established import bans for seed potatoes from third countries except Switzerland. In addition to intra-European trade, commercial ware potato imports are permitted from Algeria, Egypt, Israel, Libya, Morocco, Syria, Tunisia and Turkey. Our survey established nematode-related interceptions only in seven seed potato consignments per ten-year period. All of them originated from the Netherlands and potato rot nematode was found in none of them. Therefore, we have no data to make substantial conclusions about the effect of seed potato imports on the distribution of *D. destructor*.

Potato rot nematode infests various plant commodities. The two interceptions of *D. destructor* with ornamentals (*Polygonum cuspidatum* plants from Czech Republic intercepted by France and unspecified flower bulbs or tubers from the Netherlands intercepted by Lithuania) in our database survey confirm ability of the species to make use of alternative pathways in addition to seed- or ware potato trade. The WTO Sanitary and Phytosanitary Agreement of the General Agreement On Tariffs and Trade (GATT) stipulates that control measures must be proportional to the phytosanitary risk, developed in accordance with international standards, and must not form a disguised barrier to international trade. Since many nematodes are not readily detected by visual inspection, sampling

for laboratory diagnosis is used more frequently than for other groups of organisms (Ward & Hockland, 1996). In intra-community trade with countries where *D. destructor* has been considered of minor importance (Ward & Hockland, 1996), this procedure may be perceived as unjustified delay, especially in visually undetectable contamination levels.

The current legal regulation stipulates absence of the potato rot nematode in the seed potato, but a control mechanism for this has not been introduced, apart from the random visual tuber check. However, light infestation can easily be overlooked by a visual check since the exterior appears healthy (Kikas, 1969). A situation where the data on nematode status of soils is not systematically recorded may lead to silent build-up of pest populations in consecutive potato cultivation. In order to escape incidental contamination of seed potato production fields by uncooperative private farmers, the formation of a seed potato production region has been recommended. This was not approved back in the Soviet period, as the idea was proposed in the example of the Netherlands, Germany, Denmark, Finland etc. (Sinijärv, 1971). We suggest that current regional development initiatives could still benefit from the idea.

Conditions necessary for eradication of the potato rot nematode from infested fields still need to be verified. The importance of healthy planting material, general sanitation measures as well as crop rotation have been stressed as the main instruments for control and prevention of the spread of *D. destructor*. The nematode has been reported unable to survive in soil under cereals for 3–4 years (Sturhan & Brzeski, 1991) or for 5 years (Kikas, 1969). However, according to Estonian experience, there have been instances of contamination of pedigree seed potato nuclear stock occurring more than 5 years after the last potato crop was grown in the soil (**II**). The polyphagous habits of the nematode and its wide host range (over 70 plant species and the same amount of fungal hosts according to Manzanilla López *et al.,* 2004) let us conclude that the species can maintain a population in former potato fields for an indefinite period.

The hypothesis of an alien origin of the potato rot nematode will have to be challenged by population genetic comparisons in the future. These data would also facilitate identification of local and imported inocula, which is the basis for clarification of the infestation history in the production site as well as in the trading partner's domain.

6.2. Application of research methods for nematological studies (III, IV)

Differences in invertebrate biological, morphological and behavioural traits have major influence on the selection of methods. Potato rot nematodes remain in their host tissues as internal parasites for all life stages, whereas overwintering adults of the tested insect species (*Phyllotreta undulata* and *Meligethes aeneus)* are harboured in loose substrates like leaf litter, soil or sphagnum peat. Hence, a different approach for isolating living nematodes had to be adopted for experimental cold tolerance studies.

Extraction methods of nematodes from soil, plant material or other substrates vary greatly in efficiency. A special extraction method e.g., centrifugal flotation is required to recover inactive nematodes including egg stages from plant material (Bezooijen, 2006). Both mechanical disruption of host tissues and extraction fluid affect nematode viability, which is why direct visual extraction of small submerged host tissue samples was preferred by us over indirect nematode extraction methods. Due to these limitations, we focussed our research on motile age groups of the nematode, namely post-eclosion stages.

A detailed description of the materials and methods used for measuring the ability of nematodes to survive subzero temperatures can be found in Wharton and Rowland (1984). In the absence of a thermoelectric microscope stage for cryomicroscopic measurements, we adopted the techniques routinely used for cold tolerance studies of small insects as described in **IV**. The series of experiments on *P. undulata* one minute exposures to constant sub-zero temperatures (**III)**, indicates that the LT50 and SCP were correlated, although the former was a few degrees higher than the latter. In freeze-intolerant animals, the SCP is equal to their lower lethal temperature (LLT) or LT100 (Ramløv, 2000). Hence, materials and methods suitable for survival time determinations can be applicable for indirect estimation of SCP, when eliminating the effect of time to short exposure.

6.3. Environmental conditions influencing the survival of *Ditylenchus destructor* (V)

6.3.1. The effect of media

The results of our experiments confirm that the most favorable media for surviving moderate freezing are host plant tissues and M9 buffer. Free-living soil and plant nematodes are adapted to micro-environments with relatively high concentrations of electrolytic compounds (De Ley & Mundo-Ocampo, 2004), which prevent ice crystal formation in the buffer solution. Likewise, host plant tissues inhibit formation of inoculative ice crystals, since ice first forms in the dilute apoplastic solution by extracellular freezing. This keeps the nematodes surrounded with ice while protecting them from desiccation. Cold-induced starch-hexose conversion results in higher concentrations of glucose and its metabolites (Sowokinos, 2001) in potato tissue. These chemicals are needed by cells to synthesise trehalose, a stress protectant that interacts with, and directly protects, lipid membranes from the damage caused by environmental stresses such as desiccation and freezing (Behm, 1997). However, there is no direct evidence that potato starch metabolites contribute to the trehalose pool in nematodes.

We found the liquid media unsuitable for cold tolerance experiments in more severe cold due to ice formation. Hand picking would guarantee the provision of an exact number of viable individuals in a replication before the onset of the experiment; however, additional manipulation stress leads to an overestimation of the mortality. Likewise, overestimation of the mortality can be assumed in the case of the infested potato tissue standard samples as the population is sampled at random and a certain number of dead individuals may already be present regardless of cold as the delimiting factor. Our results have shown that the host plant tissue samples still give the most conservative mortality estimate, which is why we decided in favour of this method for future experiments.

6.3.2. The effect of subzero temperatures

Our study shows that the LT90 is highest in potato rot nematode adults (-7.4 °C) and lower in J4 and younger juveniles (-9.4 and -14.5 °C, respectively). Evidently, the most cold-resistant stages for *D. destructor* are the younger juveniles and not J4 as is the case for *D. dipsaci*

(Bosher & McKeen, 1954) or *Paratylenchus projectus* (Rhoades & Linford, 1961). There is great variation between survival ability of younger juveniles as shown by LT90 upper and lower fiducial limits (-11.1 and -28.0 °C). On the other hand, LT50 was highest in younger juveniles (-0.9 °C) and lowest in adults (-5.5 °C).

Despite differences in media and experimental methods, the LT50 for a hydrated population of *D. dipsaci* consisting predominately of J4, measured in an earlier study (Smith *et al., 2*008), falls within the confidence limits of our results with *D. destructor* J4 and adults at around -4 °C. This suggests that the contradictions between the results by Makarevskaya (1983) and Ladygina (1957) indicating lethal temperatures -4.5 °C and -28 °C, respectively, may be due to variation in proportion of different post-eclosion stages and/or set of mortality criteria (LT50, LT90 or LLT) in their experiments.

LT50 for potato rot nematode lies in a higher temperature range than for pollen beetles. The majority of the pre-winter pollen beetles survived 24 h exposure at -9 °C, but, almost all overwintered reproductive beetles died already at -9 °C. Higher lethal temperatures (LT50) are expected for nematodes as the ecological temperature extremes they have to face in their overwintering environment differ.

Our investigations established that seasonality had a significant influence on low temperature survival in selected insect species after long exposure to moderately low subzero temperature, but the ability to withstand extreme low temperatures ('intensity factor') did not depend much on season. After 24 h exposure to constant -7 °C, LT50 of *M. aeneus* did not differ significantly between new generation beetles in August, September, or February and the differences were found only with the overwintered generation in June. The effect of seasonal acclimation to soil-dwelling nematode cold tolerance still needs to be investigated.

It has been suggested that nematodes may desiccate rather than freeze in soil due to freeze concentration (Pickup, 1990; Forge & MacGuidwin, 1992). This may prevent the nematode body fluids from freezing. However, excessive concentration of extracellular and intracellular electrolytes may damage the cell membrane, leading to cytolysis (Mazur, 2004; Muldrew *et al., 2*004). Excessive osmotic shrinkage (Meryman, 1968) or membrane destabilisation (Steponkus & Lynch, 1989) may also

occur, causing cryo-injury from which the cells cannot recover. Without contact with surface water, and yet not desiccated, a nematode could employ cryo-protective dehydration (Wharton *et al., 2*003). Holmstrup and Westh (1995) suggest that a supercooled animal surrounded with ice will lose water and desiccate because the vapour pressure of the supercooled solution is higher than that of ice. Nevertheless, unlike *D. dipsaci*, *D. destructor* is unable to withstand excessive desiccation and dies at a relative humidity below 40% (Sturhan & Brzeski, 1991).

Nematodes may be able to prevent inoculative freezing by the presence of protective structures such as eggshell (Wharton, 1995). This might have been the case for a few surviving J2 at -30 °C in our experiment as the number of eggs was not counted and there is a possibility that viable individuals hatched after the termination of freezing and before assessment of mortality.

Alternatively, it might be hypothesised that the J2 and J3 exhibited another mechanism of cold survival. For example, Ladygina (1970) observed survival of the onion, clover and strawberry races of *D. dipsaci* and potato stem nematode *D. destructor* in pieces of fresh host material after cooling in a cryostat down to -28… -29 °C for several hours, but a few specimens remained viable after 5 min exposure of the infested plants to liquid nitrogen (-196 °C). Depending on the cooling rate, vitrification of the body fluids may occur in such conditions, which allows cryopreservation because water, ions and other solutes remain in their original intra- and extracellular compartments, and damage due to freeze concentration and mechanical distortion is avoided (Lee, 2010). However, since natural cooling rates do not exceed 1… 2 °C h^{-1} (Steffen *et al.,* 1989), vitrification is an unlikely strategy for body water management in overwintering plant and soil nematodes. On the other hand, we have shown that, due to the facultative endoparasitic nature of the species, host plant tissues may lower, but not prevent, reduction of population density in subzero temperatures.

Cold tolerance studies need to be continued by testing short exposure to establish the SCP of potato rot nematode. In order to evaluate the acclimation effect and rapid cold hardening, a series of laboratory experiments will be designed. Investigation on the oriented locomotion of nematodes in response to thermal and moisture gradients in winter would provide important results for determination of thermal effect on the pest population.

6.3.3 The effect of snow treatments on soil temperature and their suitability for pest control (VI)

Our experiment demonstrated that snow removal has good potential for controlling certain soil-dwelling nematode populations in the upper layers of the soil. However, the effect of this treatment in reducing the temperature in deeper soil layers probably becomes weaker due to the buffering effect. Some nematodes have long been known to be capable of seasonal vertical distribution fluctuations (Kir'yanova, 1931, 1961). Natural populations of Heterorhabditid and Steinernematid nematodes migrate to deeper layers during summer, presumably to avoid the unfavourable environmental conditions caused by high temperatures or lack of humidity (Garcia Del Pino & Palomo, 1997). Studies examining temperature gradient fluctuations have indicated that differences in the rate of thermal adaptation by a nematode can reverse the net direction it moves vertically in response to soil surface heating and cooling (Dusenbery, 1988). Whereas movement of *Ditylenchus phyllobius*, *Steinernema glaseri* and *Heterorhabditis bacteriophora* was largely random relative to the thermal surface, *Rotylenchulus reniformis* and *Meloidogyne incognita* oriented towards it (Robinson, 1994).

Our results confirm previous observations that even during the harshest winter seasons of recent years, soil temperatures rarely fall below zero. This has been shown by the soil temperature records of the Rõhu automatic weather station of the Estonian University of Life Sciences in the vicinity of Tartu (58°17'N, 26°18'E), with sensors buried to a depth of 10 cm in grassland (EMU, 2010).

Moreover, Sutinen *et al.* (1999) simulated soil temperatures at a depth of 5 cm in Finnish Lapland (67°30'N, 29°30'E), on the basis of air temperatures and precipitation data and he showed that subzero soil temperatures can hardly be expected in mild winters (min -2.2 °C with no precipitation) or moderate winters with high or mean precipitation levels (around 0 °C). Even in a harsh winter, the soil temperature curve stayed above -5 °C after snow accumulation started in high and mean precipitation schemes. The model was based on Finnish meteorological recordings with the lowest monthly minimum air temperatures in 30 years being -50 °C in January; which has never occurred in Estonia with its more southerly location. Hence, we may conclude that only the harshest winters with no permanent snow cover can naturally lower the soil temperature below

-5 °C, *i.e.*, the temperature point critical for suppressing populations of *D. destructor* in northern temperate areas.

Overwintering survival of the soil-dwelling pests is determined not only by their cold tolerance and ability to migrate vertically but also their resistance to dehydration. Benoit *et al.* (1988) have shown that water accumulated in frozen soil layers by migration to the freezing point. The result was less moisture at deeper soil depths. By inducing soil freezing, this may influence potato rot nematode survival indirectly, since the deeper soil moisture will be reduced beyond optimal limits, which possibly damages the individuals dwelling beyond the freezing zone. *D. destructor* is very susceptible to desiccation, in contrast to the related species *D. dipsaci*, a pest capable of anhydrobiosis which sometimes inhabits the aerial parts of host plants where they may experience high rates of water loss (Ustinov & Tereshchenko, 1959; Thorne, 1961; Perry, 1977; Sturhan & Brzeski, 1991; Butorina *et al., 2*006 and others). Desiccation may be an important stress responsible also for mortality of *Meloidogyne hapla* second-stage juveniles in frozen conditions (Forge & MacGuidwin, 1992). In contrast, snow accumulation has been shown to increase gravimetric soil moisture and associates with a decline in abundance of nematodes typically found in dry soils but an increase in nematodes associated with moist soil (Ayres *et al.,* 2010).

Since potato rot nematodes are only important in moist conditions, we may conclude that large scale snow removal probably has a stronger effect on potato rot nematodes than can be assumed from our initial soil temperature manipulation experiment.

As our snow compaction test was begun on a relatively warm day (air temperature +0.8 °C), on slightly molten multi-layered snow, the optimal final density was not obtained. After removal of the snowpack, burying the data loggers and returning the composite snow aggregates, the treatment effectively reduced the snowpack thickness by only 68% (from 52.6 cm to 17 cm). The resulting soil temperatures were too high to cause significant winter mortality in all except the LT50 (J2+3) level. Thermal conductivity of the compacted snow could be considerably improved if snow-treatment was initiated early in the season while the snowpack is still less than 10 cm, or in subzero temperatures, when snow crystals break instead of subliming.

Methods may be found to further improve the effect of soil temperature manipulations. One possibility would be using mulch or ground cover as recommended by Hoy (1997). Since soil under mulch remains at higher temperatures than bare soil, a rapid temperature drop can be provoked if the snow and mulch is removed immediately before the arrival of a cold front (Milner *et al., 1*992). The faster the temperature drops, the smaller the possibility that pests would have time for cold hardening or acclimatory changes of their basic cell structural components (Koštal, 2010). However, because labour costs for removing the mulch are high as it cannot be easily raked off when frozen and snow-covered, this strategy will probably be feasible only in fields limited in size (Hoy, 1997). In order to generate more conclusive results for integrated pest management strategies, this correlative study will be continued as a series of field experiments in collaboration with farms exposed to potato rot nematode infestation.

7. CONCLUSIONS

Potatoes are an economically important crop damaged by potato rot nematode in all its motile life stages. The aim of the current research was to give an overview of the anthropogenic and ecological factors influencing the spread of the nematode species. The following general conclusions may be drawn from the results of the present thesis:

1. A review of pest reports and notifications of interceptions revealed that trade volume and origin have a significant impact on the incidence of potato rot nematode in consignments. The establishment of the current distribution in international (**I**), national and subnational levels has relied primarily on random findings by the programmes targeted at other potato pathogens (**II**). The legislative restrictions against consignments originating from countries outside Europe are justified as we showed that third country ware potato imports are of higher risk. Special research programmes are necessary to establish the natural and introduced distribution of *Ditylenchus destructor* and substantiate creation of pest free areas and/or pest free production sites with the purpose of eliminating unintentional spread of contamination in the free plant market.

2. Despite the differences in bionomics and overwintering behaviour between insects and nematodes, technology specialized for insect physiological research was shown to be applicable also for the study of nematode cold hardiness. Of available methods, 24 h exposure to constant low temperatures (as in **IV)** was found most suitable for drawing ecologically relevant conclusions about the cold hardiness of potato rot nematodes. Short exposures (as in **III)** could be used for indirect assessment of SCP.

3. The choice of the cultivation media or substrate for laboratory survival experiments in moderate subzero temperatures was shown to be critical (**V**). The mortality rate ranged from 30% in potato and M9 buffer (which simulates natural ground water) to 97% in water. Inert material (gravel) did not significantly improve the viability of the nematode. To avoid over- or under-estimation of mortality, the media (as well as experimental procedures) should closely simulate natural conditions and the complex of environmental stressors.

4. Clear differences between the cold hardiness of different post-eclosion stages of the potato rot nematode were demonstrated in a series of laboratory experiments (**V**). Most resistant to extreme cold were younger juveniles J2 and J3, some of which were capable of surviving –30 °C within host tissue. This indicates the extreme ecological flexibility of the species despite the limited probability of such low temperatures occurring in the natural overwintering environment. Natural subzero temperatures alone cannot be relied upon for eradication measures. Understanding of the population dynamics of the potato rot nematode can only be achieved in the context of complex physical, biochemical and ecological processes.

5. Snow cover thickness and density manipulations resulted in significant reduction of temperatures in the upper soil layer during a cold front. The most effective treatment was snow removal, by which temperatures below -10.3 °C were achieved (**VI**). This is less than the LT50 of all the post-eclosion stages of *D. destructor* as well as LT90 of adults (**V**) which may considerably affect the winter survival of potato rot nematode population. This proves the potential of the artificial snow manipulations as an alternative nematode field suppression method.

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SUMMARY IN ESTONIAN

KARTULIINGERJA (*DITYLENCHUS DESTRUCTOR* THORNE 1945) LEVIKUT JA TALVIST ELLUJÄÄMIST MÕJUTAVAD FAKTORID

Kartulikasvatus on põhja parasvöötme kliimas läbi aastasadade taganud inimeste põhitoiduse ja sellega muutunud põllumajanduse lahutamatuks osaks. Kartuliingerjat (*Ditylenchus destructor*) teati tähtsa kartulikahjurina juba enne tema taksonoomilist eristamist sugulasliigist sibulaingerjast (*D. dipsaci*). Kuigi viimane suurem kahjustuspuhang jääb kahekümnenda sajandi keskpaika, on murranguliste üleminekuaastate tagajärjel taas näha märke kartuliingerja leviku laienemisest ja probleemi teravdumisest.

Kaasaegseid uurimistöid kartuliingerja levikut mõjutavatest antropogeensetest ja teistest teguritest pole palju. Pärast esialgseid järeldusi, et kahjustaja kontrollimiseks piisab umbrohutõrjest ja viljavahelduse järgimisest, samaaegselt kahjustuse taseme vähenemisega ning agroökosüsteemide vaesumisega kadus ka Lääne-Euroopa ja Põhja-Ameerika teadlaste huvi teema vastu. Ida-Euroopas on aga intensiivne maakasutus jõudnud alles viimastel aastatel umbrohupuhaste põllumassiivideni. Kasvanud turuhindade ja omamaise puhta algmaterjali piiratuse tõttu on kõrgekvaliteediline ja jälgitava taimetervisliku taustaga seemnekartul muutunud talunikule raskemini kättesaadavaks. Samal ajal täheldatakse aina enam intensiivpõllundusest tingitud probleemide sagenemist. Seetõttu seab jätkusuutlik põllumajandus eesmärgiks saavutada liikide kooseksisteerimise tasakaal, kus taimetoidulisi loomi (sh mikroorganisme) ei hävitata keskkonda saastavate mürkidega, vaid ohjeldatakse alternatiivsete meetoditega.

Esineb juhtumeid, mil Lääne-Euroopast toodud seemnekartuli mahapaneku järgne uus saak on osutunud kartuliingerjaga nakatunuks. Populatsioonigeneetiliste võrdluste puudumise tõttu ning eriti, kui põld omanikuvahetuse või pikaajalise söötisoleku järgselt uurimata, on saastuse lähteallikat võimatu kindlaks teha ja kartulikasvataja kannab suuri rahalisi kaotusi. Käesoleva uurimistöö üks eesmärk oli koostada ülevaade kartuliingerja levikut mõjutavatest teguritest, analüüsides Euroopa riiklike taimekaitseorganisatsioonide kümne viimase aasta kahjustaja raporteid ja kirjandusallikaid. Saadud teave toetab põhjendatud strateegiliste otsuste tegemist probleemi lahendamiseks. Analüüsist järeldub, et kartuliingerja leviku olulisemad mõjurid on kaubavahetuse mahud ja päritolu. Eestis

leitakse kartuliingerjat peamiselt teiste kartulikahjustajate (näiteks tarbekartuli baktermädanike) uuringute või seemnekartuli kvaliteedikontrolli käigus. Kui kohalik põllumajanduspoliitika seab eesmärgiks tootjate riskide minimeerimiseks kartuliingerjast vabaneda, oleks see saavutatav kahjustajavabade alade loomise või kahjustajavabade tootmiskohtade määratlemise teel. Sel juhul tuleks leida vahendid ka vastavate eriuuringute läbiviimiseks.

Teisalt mõjutavad liikide levikut keskkonnatingimused, millest parasvöötmes on sageli limiteerivaks talvised miinimumtemperatuurid. Nende mõju kartuliingerja esinemisele Eestis pole seni uuritud. Selgrootute külmakindlust on vaadeldud peamiselt putukate või teiste lülijalgsete näitel. Käesoleva töö alguses planeeriti katsed ja soetati kaasaegsed laboriseadmed, mis oleks kohased tööks ka väikesemate organismidega. Putukate külmakindluse uurimise meetodite alusel töötati välja uus algupärane metoodika kartuliingerja külmataluvuse uurimiseks. Vaatamata putukate ja nematoodide bioloogilistele ja talvitumuslikele erinevustele tõestasime, et putukate puhul kasutusel olevad tehnikad on rakendatavad ka kartuliingerja külmataluvuse uurimisel. Kuna ingerjad on fakultatiivsed endoparasiidid, kes mullas on suutelised liikuma ainult mullaosakeste vahelises vees, siis nende allajahtumispunkti vahetu määramine on komplitseeritud nende kehapinnal moodustuvate jääkristallide tõttu, mis võivad nematoode mehaaniliselt vigastada ja nad tappa. Selle tõttu osutus meetoditest sobivaimaks kartuliingerjate surevuse määramine 24tunnisel ekspositsioonil konstantsetel miinustemperatuuridel hoitud nakatunud peremeestaime kudedest võetud standardproovides. Selgrootute külmakindluse klassikaliseks indikaatoriks peetavat allajahtumispunkti on nematoodide puhul võimalik kaudselt hinnata lühiajalistel ekspositsioonidel, millega elimineeritakse ajafaktori mõju.

Kartuliingerja külmataluvuse uurimisel avaldus oluline keskkonna ehk substraadi mõju. Surevus varieerus 30–97 protsenti, jäädes madalaimaks kartuliingerjale loomuomastes keskkondades – toidutaimes kartulis ja standardses puhverlahuses M9, mis on välja töötatud vabalt elavate nematoodide lõõgastamiseks ja taastumiseks nematoloogilistes katsetes. Kõrgeim oli surevus vees. Inertaineks lisatud kruusaterad ei parandanud nematoodide ellujäävust oluliselt. Kartuliingerja liikumisvõimeliste arengujärkude isendite külmataluvuses avaldusid statistiliselt olulised erinevused. Kõige külmakindlamaks osutusid nooremad vastsed. Üksikud teise kasvujärgu vastsed suutsid peremeestaime kudedes taluda kuni –30 °C.

Siit nähtub liigi ülim ökoloogiline plastilisus, millest võib järeldada, et looduslik pakane ainufaktorina ei saa parasvöötmealadel kahjustaja populatsioone täielikult hävitada, kuid piirava tegurina mõjutab nende arvukust.

Parasvöötmes peavad mullas talvituvad selgrootud taluma raskeid keskkonnatingimusi, millega toimetulekuks on organismidel välja kujunenud mitmed ökoloogilised, füsioloogilised või käitumuslikud kohastumised. Olles laboratoorsete katsetega välja selgitanud, et vähemalt osa kartuliingerja populatsioonist on kohastunud talvitumispaiga miinimumtemperatuuriga, uuriti lumikatte paksuse ja tiheduse mõju mullatemperatuuridele. Saadud tulemuste baasil tehti järeldused kartuliingerjate võimaliku hukkumise kohta talvitumisel. Lumikatte paksuse ja tiheduse manipulatsioonid madaldasid mullatemperatuure talvise külmaperioodi ajal oluliselt. Kõige tõhusamaks osutus lumikatte eemaldamine, millega saavutati mulla pealmistes kihtides temperatuuri alanemine kuni –10,3 °C. Võrreldes seda kartuliingerja külmataluvusega, saab prognoosida, et sellest piisab valmikute 90% surevuseks, aga ka kartuliingerja kõigis teistes koorumisjärgsetes arengustaadiumites olevate isendite 50% surevuseks. Seega on mullatemperatuuridega manipuleerimine potentsiaalne tõrjevõte kartuliingerja populatsioonitiheduse reguleerimiseks.

Kartuliingerja bioloogiast põhjalikuma ülevaate saamiseks tuleks liigi populatsioonidünaamika uuringuid jätkata nii looduslike kui agrotehniliste mõjurite kontekstis.

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I

PUBLICATIONS

Impact of trade on distribution of potato rot nematode (*Ditylenchus destructor*) and other plant nematodes.

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Abstract. Scarce status reports give but a little substance for convincing conclusions about the gravity of the current problem with the potato rot nematode (*Ditylenchus destructor* Thorne 1945). This paper reviews the international experience and the survey of the most recent interception reports in member states of European and Mediterranean Plant Protection Organization. Factors influencing the distribution and invasiveness of the species are discussed.

Key words: plant health, invasion, phytosanitary measures

INTRODUCTION

Potato rot nematode *Ditylenchus destructor* Thorne 1945 has resulted in serious economic consequences in Eastern European potato production. The damage occurs at temperatures of 15–20ºC and at a relative humidity above 90% (Sturhan & Brzeski, 1991). However, according to Ilyashenka & Ivaniuk (2008), potato rot nematode has been favoured by hot dry weather and moisture deficiency in soil. Serious damage by *D. destructor* may be expected at low densities of 20–50 individuals kg $^{-1}$ of soil (Butorina et al., 2006).

ProMED (electronic reporting system for outbreaks of emerging infectious diseases and toxins) reports of plant diseases published between 1996 and 2002 show that nematodes are only minor pathogens causing about 1% of the plant emerging infectious diseases as seen in Figure 1 (Anderson et al., 2004). It was assumed that this reflects the proportion of targeted research interest rather than the actual proportion established by biodiversity inventories.

Analysing four cargo pathways, Work et al. (2005) estimated that inspections probably detected only 19% to 50% of the species being transported, depending on the particular pathway. Breakdown of European alien species data showed that a total of 1296 terrestrial invertebrates originating from other continents have established up to 2009, to which 221 cryptogenic cosmopolitan species of uncertain origin can be added. In addition to that, 964 species of European origin have been introduced to another European region (Roques et al. 2009).

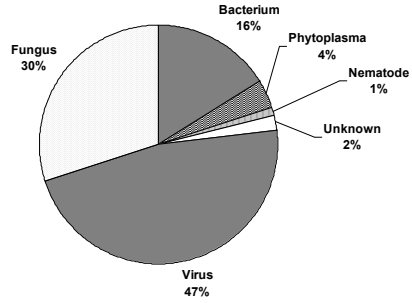

Figure 1. Characteristics of the pathogens and drivers of emerging infectious diseases of plants. By Anderson et al. 2004.

After the species differentiation in 1945, *D. destructor* has been recorded in many countries (Fig. 2), mostly from temperate regions (Sturhan & Brzeski, 1991). Wide geographic range of Ditylenchids has been interpreted as evidence of evolutionally ancient origin of the taxonomic group (Sturhan & Brzeski, 1991). The pan-European Invasive Alien Species inventory established by the EC funded project DAISIE enlists *D. destructor* as alien for the majority of Europe (Fig. 3).

Figure 2. The distribution of potato rot nematode *Ditylenchus destructor* in Europe. Source: Fauna Europaea, 2004. www.faunaeur.org.

Figure 3. The distribution of potato rot nematode *Ditylenchus destructor* in Europe. Source: Pan-European Invasive Alien Species inventory, project DAISIE, 2003. www.europe-aliens.org.

According to EPPO, potato rot nematode is reported to be present in over 70% of the member countries of the organization in the European territories (Fig. 4). Most countries have stated the pest status being of limited distribution or only a few published records. The peak of potato rot nematode damage in Eastern Europe remained in 1960–1970s (Gul'skova, 2006). Nowadays, there are serious outbreaks on ware- and seed potatoes reported in Lithuania (EPPO, 2000, 2005), Belarus (Ilyashenka & Ivaniuk, 2008; Ivaniuk et al., 2008) and Estonia (Švilponis et al., 2008). In Japan, *D. destructor* has caused damage on 18 host plants including *Brassica chinensis*, *B. oleracea*, *Capsicum annuum*, *Dendranthema morifolium*, *Cucumis sativus*, *Cucurbita moschata* and *Lycopersicon esculentum* (Nakanishi, 1979). However, serious problems occur essentially on iris and garlic crops, spreading despite the control measures (Nishizawa, 1999 as referred in EPPO, 1999). In China, it was first found parasitizing sweet potato, potato and *Mentha* (Ding & Lin, 1982). From 2004 to 2006, a root rot caused by *D. destructor* was observed on American ginseng (*Panax quinquefolium*) cultivate76d in the Beijing area and in Hebei Province in China (Zhang & Zhang, 2007). Ginseng was also found infested in Korea (Young & Seung, 1995).

Ornamentals gain importance in international plant trade both in volume and as carriers of plant pests and pathogens. In the UK, according to a database of 325 non native invertebrate plant pests, established in Great Britain between 1787 and 2004, it was recognized that 47% of all establishments occurred after 1970, with all but one of the significant post– 1970 establishments on ornamental plants (Christodoulou, 2010).

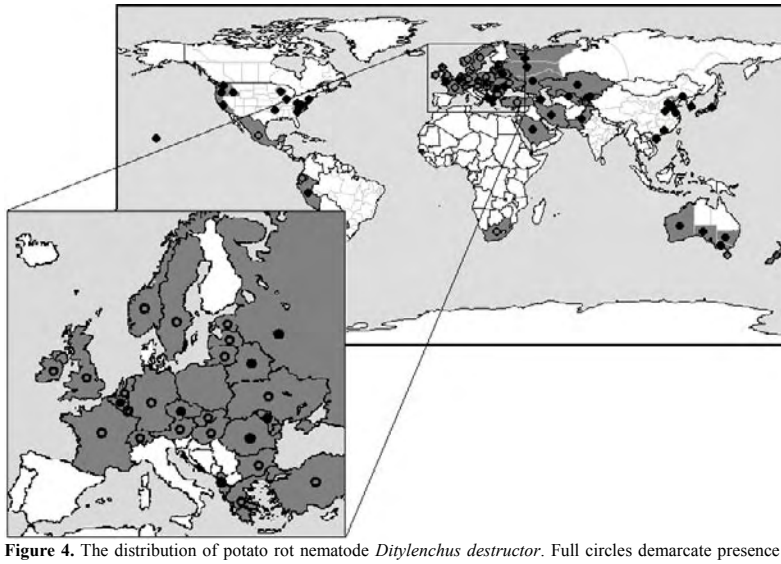

Figure 4. The distribution of potato rot nematode *Ditylenchus destructor*. Full circles demarcate presence in national or sub-national level, open circles demarcate presence only in some areas. Source: European and Mediterranean Plant Protection Organization, Distribution maps of quarantine pests for Europe, 2006. www.eppo.org.

Potato rot nematode may spread not only by planting material but also via irrigation water. It has been shown that despite of the general tendency to cause damage in colder and more humid areas (EPPO/CABI, 1997), the presence of *D. destructor* has been detected in the Iranian arid or semi-arid provinces of Semnan and Tehran, where the potato production relies heavily on irrigation. The percentage of infestation with the nematodes causing various root lesions was 87% and 54% in Semnan and Tehran respectively (Maafi et al., 2004). The percentage of field infestation to *D. destructor* was 11% in Semnan and 2% in Tehran samples (Moafi et al., 2005). Similarily, *Ditylenchus sp.* has been found infesting 28% of the samples from six major potato-producing regions in Saudi Arabia in the survey conducted from 1989–1991 (Al Hazmi et al., 1993, as cited in EPPO, 1994).

In general, plant and soil community assembly during secondary succession depends on the initial species composition, colonization from the local species pools, and on the response of the resident and colonizing species to the changing biotic and abiotic environmental conditions as shown by Kardol (2007). His data indicated that changes in the soil nematode community composition were mainly due to gradual shifts in dominance patterns in response to altered environmental conditions, even though, clear successional trends in densities of endoparasitic plant-feeding nematodes could not be determined. Polish researchers have found *D. destructor* present on dicotyledonous weeds *Anthemis arvensis*, *Bertoroa incana* and *Lycopsis arvensis* when 205 fields of common crops (20% of which were fallow) were surveyed in Wielkopolska region during 1993–1994 (Kornobis & Wolny, 1997, as cited in EPPO, 1998).

Conversely, the general quarantine regulations against the potato rot nematode in European territories have been abolished, as it was removed from EPPO A2 quarantine pest list in 1984, because of its very wide distribution in the region (EPPO, 1987, 1988). Nowadays, *D. destructor* is regulated under the EU Plant Health

Directive (2000/29/EC) Annex II/A2, e.g. it is enlisted among harmful organisms whose introduction into, and whose spread within, all member states shall be banned if they are present on flower bulbs and corms of *Crocus*, *Hyacinthus* , *Iris*, *Trigridia*, *Tulipa*, miniature cultivars and their hybrids of the genus *Gladiolus*, intended for planting, and potato tubers, intended for planting. Due to these rules, the findings of *D. destructor* on other ornamental host species are not subjected to notification nor are they actionable. Apart from general plant health regulations, there are no intergovernmental programs targeted against the potato rot nematode nor there is evidence on allocations for specific measures.

We assume that distribution and spread of *Ditylenchus destructor* in potato growing areas is favoured by several anthropogenic factors: trade volume and market demand, absence of legislative measures and targeted research programs, cultivation practices and availability of control options. Literature review and data survey were conducted to summarize the existing international and national experience with the pest within the context of current agricultural trends.

MATERIALS AND METHODS

Data was retrieved from the '*EPPO Reporting Services'*, which are regularly published by the European and Mediterranean Plant Protection Organization (EPPO), where notifications of noncompliance (detection of regulated pests) are reported by the member countries. The analysis considered all the pest interceptions having occurred in the eleven years from 2000 to 2010. According to the availability of data, the reports of a total of 28 countries were considered. According to the United Nations geoscheme (United Nations Statistics Division, 2011), the countries were separated into following regions:

- Western Europe: Austria, Belgium, France, Germany, the Netherlands, Switzerland,
- Northern Europe: Denmark, Estonia, Finland, Ireland, Latvia, Lithuania, Norway, Sweden and the United Kingdom
- Southern Europe: Greece, Slovenia and Spain
- Eastern Europe: Bulgaria, Czech Republic, Hungary, Poland and Russia
- Northern Africa: Algeria and Tunisia
- Western Asia: Cyprus, Israel and Turkey

The following variables were surveyed:
1) Relative importance of different nematode families and major species intercepted;
2) Relative importance of associated commodities and
3) Origin classified into the regions according to the categories of the United Nations geoscheme.

The survey data on most important plant parasitic nematodes was collated in order to calculate the relative indices on establishment success. Comparison of the indices by the nematode groups was performed by *Chi-square* test.

RESULTS AND DISCUSSION

Altogether, 879 reports were reviewed with the details of 1088 nematodes, 318 of which concerned nematode infested bonsais, 312 plants for planting, 114 aquarium plants, 113 wooden packaging materials, 97 ware potatoes, 28 bulbs (tubers) of ornamentals or vegetables excluding potatoes, 26 raw wood, 16 soil or growing medium, 7 seed potato, 6 seeds and 6 other materials.

Analysis on relative importance of nematodes in the international plant trade shows that potato rot nematodes were present in about 3% of 221 potato lots intercepted with nematode contamination whereas 90% of interceptions were caused by potato cyst nematodes. Nevertheless, *D. destructor* was among the ten best

recognised phytonematodes in the international plant trade, although it has been identified to species level only in 8 cases while *Globodera rostochiensis* has been reported in 69 and *Bursaphelenchus xylophilus* in 60 cases for the same period. Nematode families (sub-families) intercepted in international consignments during phytosanitary checks are presented in Figure 5.

Of the 1088 nematode interceptions that occurred between 2000 and 2010, the vast majority concerned nematodes related to bonsais or aquatic plants. The origin of alien nematodes differed significantly depending on the associated commodity (comparing potatoes with timber $\chi 2$=96.35; P=0.000 or with bonsais $\chi 2$=334.37; P=0.000). The nematode species arriving with bonsais and wood packaging material originated predominantly from Asia (96.7 and 76.9%, respectively) whereas those arriving with timber and logs came from Russia (47.9%) but also through intra-European exchanges. In addition, the diversity of nematodes carried by bonsais was significantly higher than that of the nematofauna carried by potato or timber products ($\chi 2$=7.26; P=0.007 and $\chi 2$= 7.61; P=0.005 respectively). Majority of the ware potato consignments in our survey originated from Europe (66 interceptions). To the lesser extent, other continents contributed: Western Asia with 25 consignments and Northern Africa with 3 consignments. Potato rot nematode was found by Bulgaria in 2 consignments from Turkey and by Lithuania in 4 consignments imported from the Eastern European countries (Belarus, Moldova, Poland, Hungary).

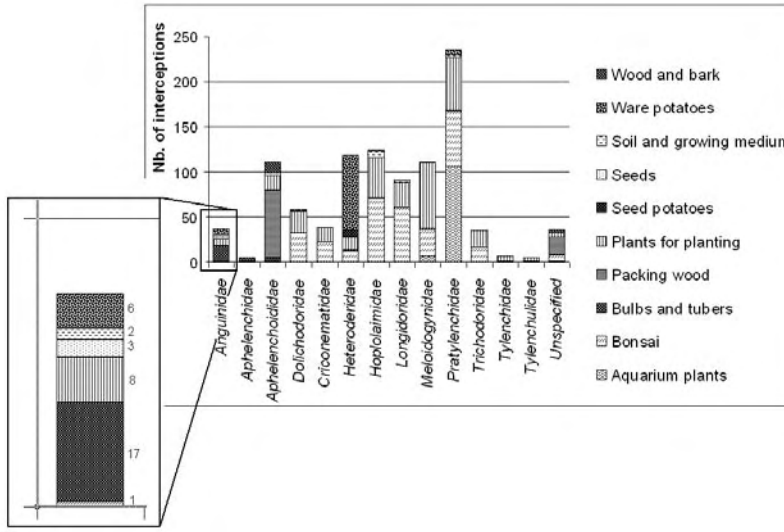

Figure 5. Nematode groups associated with major commodities intercepted by EPPO member countries from 2000– 2010. Genus *Ditylenchus* belong in the family Anguinidae.

Of the 1181 consignments, 678 were intercepted by the Western European countries, including 245 with bonsais, 186 of plants for planting and 167 of aquarium plants. In Northern Europe, 362 consignments were found infested with the major risk commodities being ware potatoes, wooden packaging material and plants for planting (138, 115 and 44 interceptions respectively). In this region, bonsais were reported as carriers of plant nematodes only by the United Kingdom, whereas aquarium plants by Denmark. The major nematode concerns for Eastern and Southern European countries were associated with ware potatoes (66 interceptions of total 111 by the former) and raw timber (5 reports of total 9 by the latter). Western Asia contributed with 16 interception

reports and Northern Africa 5 reports. Intercepted seed potato was exclusively of Western European origin. Only potato cyst nematodes (*Globodera rostochiensis* and *G. pallida*) were found in seed potatoes.

The interception data analysis shows clearly that the origin of the consignments plays a critical role in nematode incidence rate. Our survey results support the theory by Blumler (2006), indicating that the number of introduced species present correlates with the number of different early trade routes, frequency of intentional introductions, trade volume (particularly of unprocessed agricultural products) and the size of region receiving introductions. According to EUROSTAT database, yearly ware potato trade in Europe from 2000– 2010 totals in over 900 to 1600 billons of Euros in value. Considerably smaller volume is imported from Western Asia or Northern Africa, with the same indicator ranging roughly between 50 and 100 billions of Euros annually. Ware potato infestation rate with all nematodes detected by our survey was shown larger in consignments originating from Europe (70%) in comparison to third countries (30%). These data are roughly comparable to *D. destructor* findings with no significant differences ($\chi 2 = 0.85$; $P = 0.35$). Recognizing the fact that international potato trade with third countries averages only 10% of the intra-European trade values, we may conclude that the import material is of significantly higher pest incidence ergo higher phytosanitary risk ($\chi 2 = 6.02$; $P = 0.014$).

The correlation between trade volume and the number of invasive species incursions has been reported by many authors (Levine & D'Antonio, 2003; Blumler, 2006; Švilponis et al., 2010). In case of weeds, Forcella & Wood (1984) envisaged a geographical rule that invasion success correlates with the size of a species' natural distribution. It has been suggested, that the biological traits enabling some species to spread across their native territory also facilitate their invasion to new areas (Roy et al., 1991). Due to the unknown history in case of potato rot nematode, the foreign nature of the species in Europe can not be confirmed and it should be categorized as cryptogenic. Hence, the invasion success can not be estimated without respective population genetic evaluations.

Comparison of the distribution maps (Fig. 2, 3 and 4) shows the limitations of information for establishing the present range of the species. Apart from trade-policy, reasons may be the absence of recent findings in regional level (e.g. in Western Europe) or lack of nematology experts (e.g. in Eastern Europe). In an attempt to explain the variation in the presence of *D. destrucor* in Europe, one may appeal to the ecological principle of Competitive Exclusion (Speight et al., 2008). Evidently, homogeneous habitats with relatively few niches available support fewer species than heterogeneous ones, since the species rich habitat allows for more coexistence by enabling them to partition resources within the habitat and avoid interspecific competition. Mosaics of mixed seminatural habitats were characteristic to Eastern European agricultural landscapes, whereas human impact has been more disruptive in Central and Western European rural areas. Such habitat mosaics are also considered to reduce the probability of extinction of rare species.

Potato rot nematode infests various plant commodities. The two interceptions of *D. destructor* with ornamentals (*Polygonum cuspidatum* plants from Czech Republic intercepted by France and unspecified flower bulbs or tubers from the Netherlands intercepted by Lithuania) in our database survey confirm the species ability to make use of alternative pathways in addition to seed- or ware potato trade. The WTO Sanitary and Phytosanitary Agreement of the General Agreement On Tariffs and Trade (GATT) stipulates that control measures must be proportional to the phytosanitary risk, developed in accordance with international standards, and must not form a disguised barrier to international trade. Since many nematodes are not readily detected by visual inspection, sampling for laboratory diagnosis is used more frequently than for other groups of organisms (Ward & Hockland, 1996). In intra-community trade with countries where *D. destructor* has been considered of minor importance (Ward & Hockland, 1996), this procedure may be perceived as unjustified delay, especially in visually undetectable contamination levels.

The current legal regulation stipulates absence of the potato rot nematode in the seed potato, but control mechanism for this has not been introduced, apart from the random visual tuber check. However, light infestation can easily be overlooked by visual check since the exterior appears healthy (Kikas, 1969). Situation where the data on nematode status of soils are not systematically recorded may lead to silent build-up of pest populations in consecutive potato cultivation. In order to escape incidental contamination of seed potato production field by uncooperative private farmers, formation of seed potato production region has been

recommended. We suggest that current regional development initiatives could still benefit from the idea (Švilponis et al., 2008).

Conditions necessary for eradication of the potato rot nematode from infested fields still need to be verified. The importance of healthy planting material, general sanitation measures as well as crop rotation have been stressed as main instruments to control and prevent the spread of *D. destructor*. The nematode has been reported unable to survive in soil under cereals in 3–4 years (Sturhan & Brzeski, 1991) or in 5 years (Kikas, 1969). However, according to Estonian experience, there have been instances with contaminations of pedigree seed potato nuclear stock occurring over 5 years after last potato crop grown in the soil (Švilponis et al., 2008). The polyphagous habits of the nematode and wide host range let us to conclude that the since the species is able to maintain the population in formerly contaminated fields for an undetermined period, pest free area is the only reliable control measure to guarantee safe plant material in international plant trade.

Hypothesis of alien origin of the potato rot nematode will have to be challenged by population genetic comparisons in the future. These data would also facilitate identification of local and imported inocula, which is the basis for clarification of the infestation history in the production site as well as in the trading partner's domain.

CONCLUSIONS

By reviewing the pest reports and notifications of interceptions, it was revealed that trade volume and origin have a significant impact on the incidence of potato rot nematode in consignments. The establishment of current distribution in international, national and subnational levels has relied primarily on random findings by the programs targeted at other potato pathogens. Legislative restrictions against consignments originating from countries outside the Europe are justified as we proved the third country ware potato imports of higher risk. Special research programs are necessary to establish the natural and introduced distribution of *Ditylenchus destructor* and substantiate creating pest free areas with the purpose to eliminate unintentional spread of contamination in free plant market.

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PLANT PARASITIC DITYLENCHIDS IN ESTONIA

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Abstract

The first crop losses caused by potato rot nematode (*Ditylenchus destructor* Thorne) in Estonia have been reported since 1950s. Today ditylenchids are considered common but generally neglected as harmful pests with the exception on certain crops. This has resulted in serious consequences for seed potato producers in several cases. The current state of the national research and surveillance is reviewed and pest management strategies are discussed to provide general recommendations for overcoming such difficulties.

Key words: *Ditylenchus*, potato rot nematode, pest free place of production.

Introduction

Potato rot nematode, *Ditylenchus destructor* Thorne has been recognised as a serious pest of potato in Estonia since 1950s. During the last peak of damage severity, it was evaluated to cause greater crop losses than potato cyst nematode, *Globodera rostochiensis* Wollenweber /Krall, 1974/. The distribution of the species is wide /Krall, 2001/. The survey in the middle of 1950s found infestations in Tartu, Harju, Kohtla-Järve, Valga and Pärnu regions /Krall, 1958/. Potato rot nematode causes dry rot of tubers that can turn into soft rot in time. There are not many investigations on biology and ecology of potato rot nematode in the world. Somewhat better is the situation with stem and bulb nematode, *Ditylenchus dipsaci* Kühn. This paper reviews critically the available information on plant parasitic ditylenchid species in order to clarify the pest status in the country.

Materials and Methods

Stem and bulb nematode, *D. dipsaci* was reported to be introduced to Estonia annually by the international trade of onion seeds already in the beginning of the 20th century /Zolk, 1935/. Stem nematodes (under the name *Tylenchus dipsaci* Kühn) were known to infest stems of grasses and clover as well as potato and onion. However, unlike potato cyst nematode, *G. rostochiensis*, seed and leaf gall nematode, *Anguina tritici* Steinbuch and strawberry crimp nematode, *Aphelenchus fragariae* Ritzema Bos they were not considered to be of quarantine significance as they did not seem to survive for establishment in the region.

First systematic research records on potato rot nematode, *D. destructor* in Estonia originate from prof. Eino Krall who started field survey of potato nematodes in Tartu region in 1954. The survey was extended for following years up to 1959 in the territory of whole republic /Кралль, 1959/. In total 553 samples were taken from 63 localities, and nematode species were determined in 87.7% of the samples. Survey samples included aerial parts, roots and tubers as well as rhizosphere soil of potato plants. Nematodes of plant tissues were extracted by Baermann funnel method while initially 10 cm^3 soil samples were filtered through four 80 μm sieves. In order to increase efficiency, direct observation method of 1g soil samples in water was applied in the latter stages of the study. Nematodes were fixed by formalin and transferred through glycerine-ethanol method to permanent glycerine-gelatine mounts. Potato rot nematode was detected only once in aerial parts of plant, but it was a common species among the nematofauna of potato tubers, attaining maximum population only by the end of winter storage period. For detection of tuber infestation, Krall (1974) used the following method: after soaking the tubers in water for a few hours, decanted suspension was pipetted on the clean glass slide for direct observation by dissecting microscope. Alternatively the suspension can be decanted from pieces of white tuber tissue detected under the potato peelings or between rot-affected and healthy tissues.

Prof. Krall and his team in the Institute of Zoology and Botany of Estonian Academy of Sciences acted many years in functions of national reference centre for nematode diagnostics. They received samples from producers consisting of some tens of grams of soils and parts of fibrous roots of symptomatic plants, or aerial parts if distortion of aboveground organs occurred /Krall, 1965/.

The status of *D. dipsaci* complex has not been subjected to thorough studies, partially because of the difficulties to determine the phylogenetic species or trophic races /Чижов, 2006/. There have been indications of damage on onion in Peipsi lake region /Krall, 1965/, daffodils in Tartu /Кралль, 1985/, red clover in Tartu and Pärnu counties /Krall, 1965/, white clover in Tartu region Nõo and lucerne in Hiiumaa Kassari /Krall, 2001/, on tulips and in 1955 on rye /Krall, Luik, 2000/. In addition to this, there are records on findings of ditylenchids in natural plants and weeds. Root-gall nematode, *D. radicicola* Greeff has been indicated to be under pathogenecity studies to determine the damage potential for barley /Кралль, 1965; Krall E., Krall H., 1968/. There have been no records of rice stem nematode, *D. angustus* (Buther) Filipjev and *D. mycelophagus* Goodey in Estonia /Krall, Luik, 2000/.

In the middle of the 20 century progress was made in the field of nematode ecology. Some field and laboratory research has been performed to investigate behaviour of other phytopathogenous nematodes like *Globodera rostochiensis* /Mägi, 1983/, *Heterodera trifolii* Goffart /Рийспере, Роосма, 1985/ *H. avenae* Wollenweber /Мяги, 1989; Krall, Müür, 1999/, *Paranguina radicicola* Kirjanova /Krall E., Krall H., 1968/. However, there are no recent ecological studies on ditylenchids in Estonia.

Estonian phytonematology has been focussed mainly on faunistic studies by professor Krall and his team. Recently cooperation with nematologists from other countries has determined the phylogenetic relationship between 23 populations of *D. dipsaci* and developed species specific primers for identification of *D. dipsaci sensu stricto* /Subbotin et al., 2003; 2005/. The higher education quality assessment has

evaluated prof. Krall's work in the structures of Tartu University and concluded the situation in 2000 satisfactory /Nikinmaa et al., 2000/. The valuable and extensive nematode collection of the Institute of Zoology and Hydrobiology has not been assimilated into zoological collections of Tartu University nor to the study collections of Estonian University of Life Sciences. The critical review of the collection has been performed to compile a databank /Krall, 2000/. Unfortunately, the majority of the nematode slides are deteriorated by today, which indicates the need to start using a different methodology for preparation of permanent mounts.

Currently no data is published on the quantity of national surveillance samples taken during the Soviet period and found positive for *D. dipsaci* or *D. destructor*. National Plant Protection Board started to promote rot-nematode-free seed potato production in 1960s /Kikas, 1969/. They recommended a complex of control measures including a general detection survey of *D. destructor*, practice of crop rotation with potato-free period of 4–5 years, pest free seed potato, and weed control.

Distribution level of potato rot nematode was estimated by Randalu (1971) based on the seed potato quality analyses in spring 1970 by the National Plant Protection Board which resulted in 50% infection by *D. destructor*. Since 1973 there have been records on the regular work of certification commission for seed potato super elite and elite categories /Rosenberg, 2002/. In 1984 a new seed potato production scheme was enforced by Agricultural Industry Assembly to use only in vitro basic material. By 1992 100% of super elite originated from in vitro clone cultures. Since 1994, EVIKA laboratory lost the possibility to multiply the protected varieties that lead to sudden decrease of certified seed potato production area from 1300 ha to 76 ha in 1999 /Rosenberg, 2002/.

Table. Results of national surveillance sampling (reason indicated) and findings of *Ditylenchus* destructor (DITYDE) and *D. dipsaci* (DITYDI)

YEAR	Seed potato tuber analyses			Monitoring of ware potato bacterial diseases		Tuber analyses on producer's request	
	No of samples	Positive DITYDE	Positive DITYDI	No of samples	Positive DITYDE	No of samples	Positive DITYDE
2003	97	0	0	unknown	6	unknown	0
2004	80	0	0	unknown	0	unknown	0
2005	151	2	0	unknown	2	unknown	0
2006	195	0	0	357	3	unknown	0
2007	156	0	1	unknown	1	3	1

Data source: Estonian Plant Production Inspectorate, annual plant health surveillance reports, www.plant.agri.ee

Phytosanitary regulation under the Seed and Propagating Material Act has set the requirement of soil freedom from *D. destructor* in seed potato production. Annually, nematode freedom shall be verified by four 250 ml soil samples per hectare taken by an authorized sampler and analyzed in Plant Health Laboratory of Estonian Agricultural

Research Centre. However, as indicated in the current regulation, the soil sample is used for potato cyst nematode analysis only /Seemnekartuli kategooriad …, 2006/. Official tuber quality check by inspectors of Estonian Plant Production Inspectorate is a random sampling, a ratio of which depends on the lot size: one sample of 200 tubers shall be taken per lot size up to 20 t, two samples per 60 t, 3 samples per 100 t and from there on additional one sample per every 50 t /Seemnekartuli kategooriad …, 2006/. Table presents official analysis results on ditylenchid pests in the course of Plant Production Inspectorate national surveillance activities in recent years by plant health laboratories.

Results and Discussion

Nematology research in Estonia started not before 1930s. Even then the lack of taxonomic and ecological knowledge made the agricultural stakeholders not to adopt official phytosanitary measures for ditylenchids. Activation of research and national surveillance in 1960–70s indicates serious economic consequences occurring at that period. Krall /Кралль, 1959/ reports regular 6–7% infestation of potato rot nematode in storages and suggests the pest was widely distributed in continental part of Estonia already in 1953 when first notifications of pest damage appeared. Kikas (1969) illustrates the pest severity by examples from Tõrva region where about 50% of tuber crop was infested by the end of growing season when healthy (symptomless) seed was planted on a possibly contaminated soil. Next year there were 80–90% tubers infested on the same plot planted with tubers from the previous yield.

Mass and single clone selection and indexing have been systematically employed as quality measures in Estonian seed potato production due to the possibility of export since 1930s /Võsaste, 1971/. Production experiments for disease eradication were started by Estonian Research Institute of Agriculture by E. Kaarep in 1958 /Jaanvärk, 1966/. During the early years mainly fungal diseases and potato viruses were targeted. Sinijärv (1971) has discussed how potato rot nematodes were distributed by the means of contaminated seed potatoes. Due to higher procurement price of ʻJõgeva kollane', this table potato variety production was forced. We may guess that abnormal demand and poor supply created the market for low-quality seed. Sinijärv (1971) admits that all producers of ʻJõgeva kollane' elite seed had soils infested with potato rot nematode and instructs for usage of pest-free fields for seed production. In order to escape incidental contamination of seed potato production field by uncooperative private farmers he advises formation of seed potato production region by the example of the Netherlands, Germany, Denmark, Finland etc. Although this was not approved back in Soviet period, we urge that current regional development initiatives could still benefit from the idea.

Many authors have stressed the importance of healthy planting material, general sanitation measures like removal of infested tubers from the field and storage as well as crop rotation as main instruments to control and prevent the spread of *D. destructor*. Sturhan and Brzeski (1991) declared crop rotation in 3–4 years with cereals and maize together with weed control as exceptionally effective. Estonian sources claim the nematode is unable to survive in soil under cereals for 5 years /Kikas, 1969, Hiiesaar, Metspalu, 2002/. Nevertheless, according to a chief inspector Elsa Aru (personal communication) there have been instances with contaminations of pedigree seed potato nuclear stock occurring at least 5 years after last potato crop grown in the soil. The

polyphagous habits of the nematode and wide host range (over 70 plant species and the same amount of fungal hosts according to Manzanilla-López et al., 2004 allow) us to notify that the species can be able to maintain the population in former potato fields for an undetermined period.

Fluctuation of potato rot nematode population density is proposed by Gul'skova /Гульскова, 2006/. She reports the peak of potato rot nematode damage in the former Soviet Union remaining in 1960–70. The problem in Estonia was alleviated by starting to use in-vitro cultured basic material, informing state farms' agronomists and seed potato producers of potential threats and by changing the varieties to more resistant or tolerant ones. In the middle of 1990s, neither stem nor potato rot nematodes were considered among 55 major crop pests and 50 diseases for annual national prognosis survey /Taim, Soobik, 1994, 1996/. Re-appearance of the problems now, more than ten years later, indicate that Estonian soils may have been carrying low level of infestation ever since it was first discovered in the middle of the 20th century. Collapse of the soviet seed potato propagation and national surveillance system in 1990s may have been another serious setback from potato health point of view. More studies are needed to investigate causative factors of such population build-ups as well as multitrophic relationships with ditylenchids in various soil types.

Some countries have recommended soil fumigation to destroy harmful nematofauna. Soil solarization may be considered ineffective due to lack of sun radiation intensity in our latitude. In Estonia, karbation has been recommended as soil disinfectant back in 1960–70s /Krall, 1965; Kikas, 1969/. Reluctance of plant producers to adopt this as a common practice due to its toxicity to plants has helped to preserve natural biological diversity in soils /Krall, 2000/. Hiiesaar and Metspalu (2002) admit lack of effective control measures against potato rot nematode and suggest agrotechnological methods. Currently there is no research available on damage threshold or biodiversity assessment in Estonian conditions for advisory purposes to guide integrated control strategies.

Many countries with agricultural sector being of major importance in the economy have targeted ditylenchids by legislative measures. According to Lehman (2004), it has been an increasing trend among countries as *D. dipsaci* was reported to be regulated in 23 countries in 1982 while by 2000 the number has doubled – 58. The same figures for *D. destructor* are 12 and 53, respectively. Hockland and her co-workers suggest this is because those species are easily transported in the international plant trade due to their endoparasitic nature /Hockland et al., 2006/. The fact that ditylenchids in Estonia (as well as in Europe in general) are listed as harmful organisms only in the case of plant propagation material means they are not considered of quarantine but regulated non-quarantine significance. This status is still incomprehensible for government officials in many countries, and may give a rise to some international phytosanitary disputes under International Plant Protection Convention or WTO Agreement on Sanitary and Phytosanitary Measures.

The current legal regulation defines the requirement of absence of the potato rot nematode in the seed potato, but control mechanism of this has not been introduced, apart from the random visual tuber check. However, light infestation can easily be overlooked by visual check since the exterior appears healthy /Kikas, 1969/. As

indicated in Table, all official samples of ditylenchids in recent years have been taken due to other tasks and no potato rot- or stem and bulb nematode monitoring has been carried out. In fact, data on nematode status of soils are not systematically recorded, which may lead to silent build-up of pest populations in consecutive ware potato cultivation. There have been cases of rejection of seed potato lots due to infestation by *D. destructor*. This causes the producers to lose their annual income as well as initial investments as they cannot market the contaminated yield by the purpose. Ware potato producers with identified infestation are advised by national inspectors not to grow potatoes in their fields. Although there is a legal option for producers to apply for a Pest Free Area or Pest Free Place of Production according to Plant Protection Act, no steps for putting this into effect seem to have been taken.

Conclusions

With respect to restriction of spread of potato diseases, a requirement for registered ware potato producers was enforced since the 1st of January 2006 to replace 20% of planting material with certified seed. Nevertheless, using not-certified 80% of the planting material, if not analyzed on producer's own initiative is the source of constant hazard. We conclude that Estonian agricultural policy should set an aim to eradicate potato rot nematode or stem and bulb nematode along with trying to achieve at least production site freedom for plant propagation material. This could be the only effective way to preserve the remaining nematode-free fields and to support competitiveness of Estonian crop seed and seed potato producers on international markets. For ware potato production, a systematic survey of damage threshold levels per soil type should be performed.

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III

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Factors affecting cold hardiness in the small striped flea beetle, *Phyllotreta undulata*

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Key words: acclimation, cold tolerance, food plant, lower lethal temperature, seasonal change, starvation, sub-zero temperatures, supercooling point, Coleoptera, Chrysomelidae

Abstract

The striped flea beetle, *Phyllotreta undulata* Kutschera (Coleoptera: Chrysomelidae), is a pest of cruciferous crops. It overwinters as an adult. During winter in northern European countries, such as Estonia, it is subject to sometimes severe temperatures that may fluctuate daily, over the season, and between seasons. The objective of this study was to investigate factors that affect its cold hardiness. In a series of five experiments, the effects of food plant, starvation, and acclimatization on the beetles' ability to supercool and survive exposure to sub-zero temperatures was investigated. The supercooling points (SCP) of overwintered beetles field-collected from white mustard and Indian mustard differed from those caught from white cabbage and oilseed rape, but these differences disappeared after a 4-day period of starvation at room temperature, indicating that gut content probably influences the potential to supercool. The duration and temperature of acclimation affected SCP in overwintered beetles. The decrease in SCP was more rapid at 22 °C than at 0 °C, probably because of faster dehydration and gut evacuation at the higher temperature. Acclimation at 0 °C for a week increased the ability of overwintered beetles to survive sub-zero temperatures, lowering both SCP and lower lethal temperature (LLT_{50}). Some pre-freeze mortality occurred; SCP and LLT_{50} were correlated but the latter was a constant 3 °C higher than the former. The SCP of field-collected pre-winter beetles decreased gradually during the autumn. It also decreased when field-collected pre-winter beetles were acclimated at 0 °C in the laboratory, attaining its lowest level after 18 days. *Phyllotreta undulata* is well-adapted to unstable and sometimes severe winter conditions; its high potential to supercool enhances its cold hardiness and ability to survive short periods at sub-zero temperatures although it cannot survive freezing of its body fluids.

Introduction

Flea beetles (Coleoptera: Chrysomelidae) inhabit a wide range of environments where cruciferous plants grow, including fields, gardens, and uncultivated areas. In Estonia, six species of flea beetle are found on cruciferous crops, the most common and damaging of which is the small striped flea beetle, *Phyllotreta undulata* Kutschera (Hiiesaar et al., 2003). Although flea beetles colonize crops every year, their population densities vary widely between years (Hiiesaar et al., 2004). Flea beetles have been much studied (e.g., Burgess, 1977; Nielsen, 1977, 1996; Kinoshita et al., 1979; Wylie, 1979; Lamb, 1983; Andersen et al., 2005) but there is little information on their cold hardiness, i.e., their ability to survive exposure to low temperatures (Zachariassen, 1985; Lee, 1991).

Flea beetles are univoltine. They overwinter as adults, usually outside fields, in margins, in hedgerows, and beneath shrubs, although some find shelter within fields in leaf litter, in stubble, or in grassy areas (Andersen et al., 2005; Ulmer & Dosdall, 2006). Physical conditions at their overwintering sites may fluctuate daily, seasonally, and between years. As they do not burrow deep into

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the soil, their survival over winter depends on winter temperatures and the depth of any snow layer. In northern European countries, like Estonia, they can experience different conditions in different years; winters with a deep snow layer may alternate with snowless winters and temperatures may vary widely. In some years, they may be subjected to sub-zero temperatures even in April and May, after their emergence from hibernation.

Insects have developed various behavioural and physiological strategies to survive severe winter conditions (Lee, 1989, 1991; Block, 1991, 1996; Bale, 1996; Danks, 1996). In preparation for winter, insects may stop feeding, seek a favourable microhabitat, empty their digestive system, undergo dehydration, and synthesize polyols and proteins to protect them from freezing (Zachariassen, 1985; Sømme, 1999).

Many insects rely on supercooling to lower the temperature i.e., the supercooling point (SCP), at which their body fluids freeze. The cold hardiness of an insect species has been widely assessed by determining the SCP. However, this is not a reliable indicator for all species because, in some, mortality occurs at temperatures above or below their SCP (Bale, 1993; Carrillo et al., 2005). Thus, for example, SCP is an accurate predictor of cold hardiness for *Coccinella septempunctata* L. (Nedvěd et al., 1995) but not for *Mamestra configurata* Walker (Turnock et al., 1983). Many insects, when exposed to cool temperatures for days or weeks in the autumn, become acclimated, and their cold hardiness increases aiding their survival during cold periods (Bale, 1989; Sømme, 1996; Renault et al., 2002).

The objective of this study was to investigate factors that may affect cold hardiness of *P. undulata.* In a series of five experiments, we examined the effects of food plant, starvation, and acclimation on their ability to supercool and survive exposure to sub-zero temperatures.

Material and methods

Source of beetles

The flea beetles were field-collected from crops growing in the vicinity of Tartu, Estonia (58°23'N, 26°41'E). Those used in experiment 1 were collected in June 2006 from four cruciferous crops (*Brassicaceae*), namely white cabbage (*Brassica oleracea* var. *capitata f. alba* L.), white mustard (*Sinapis alba* L.), Indian mustard (*B. juncea* L.), and oilseed rape (*B. napus* var. *oleifera* L.). The crops were isolated from each other spatially; the landscape was fragmented by forest and cereal fields, and the distance between crops was about 2 km. Beetles used in experiment 2–5 were collected from white cabbage. Two generations of beetles were used. In experiment <2–3, overwintered beetles collected in early June 2007 were used, whereas in experiment 4 and 5, their progeny were used, pre-winter beetles collected in August–September 2007.

Measurement of supercooling point

Supercooling point was measured using a copper-constantan thermocouples-thermometer (RS-232, Data logger Thermometer; TES Electrical Electronic, Taipei, Taiwan). Low temperatures (down to −30 °C) were attained by deep-freeze Haier HF-103. Prior to supercooling, beetles were cooled to a low temperature (0 to −2 °C) to immobilise them. Each was then fixed individually to the top of the thermocouple with a thin layer of vaseline, and sealed in a plastic tube. The tubes were placed in a cotton-lined container and transferred into the freezing chamber at −30 °C. As cooling rate can affect the SCP, we chose a standard rate of 1 °C min^{-1} (Salt, 1966; Merivee, 1978; Cannon, 1983). The temperature at which latent heat was released during freezing was taken as the SCP of the beetle. Since gender does not affect SCP (Muljar et al., 2007), mixed sex beetles were used in all experiments. The number of beetles used per experiment varied and is given below.

Experimental procedures

Experiment 1: Effect of food plant and starvation on SCP of overwintered beetles

Overwintered flea beetles, collected from white cabbage, white mustard, Indian mustard, and oilseed rape, had their SCPs determined either immediately after collection (non-starved), or after being kept in the laboratory without food at room temperature (ca. 22 °C) for 96 h (starved) before their SCPs were determined.

Experiment 2: Effect of duration of acclimation at various temperatures on SCP of starved overwintered beetles

Overwintered beetles were divided into two groups with about 120 individuals in each. One group was kept at room temperature (ca. 22 °C), while the other was kept at 0 °C. Neither group had access to food, i.e., they were starved. After 1, 2, 3, 7, and 8 days, a sample (see Table 3) of beetles was removed from each group, and their SCPs determined.

Experiment 3: Effect of sub-zero temperatures on SCP and mortality of non-acclimated and acclimated overwintered beetles

Overwintered beetles kept at 0 °C for 7 days in the laboratory (acclimated) were compared with overwintered beetles used directly after collection from the field (non-

acclimated). Groups of 20–56 acclimated or non-acclimated beetles were each enclosed in a 25-ml plastic tube and cooled in the deep-freeze to a temperature of −4, −6, −8, −10, −12, −14, −16, or −18 °C, at a cooling rate of 1 °C min^{-1}. They were kept at these temperatures for 1 min and then re-warmed to room temperature at the same rate as they had been cooled. The cooling profile was monitored with a thermocouple placed inside each tube with the beetles. Mortality was determined after 2 h; any beetle not moving or responding to tactile stimuli was considered dead. The experiment was replicated four times. To determine whether the SCP of the beetles is a reliable indicator of their cold tolerance we compared lower lethal temperatures (LLT_{50} = temperature at which 50% of beetles died) with mean SCPs for non-acclimated and acclimated beetles.

Experiment 4: Effect of collection date on SCP of pre-winter beetles

Pre-winter beetles were collected weekly on five dates from 15 August to 14 September 2007. SCPs of 10 beetles were determined on each collection date. The assessment on 14 September was the final one, because 1 week later all beetles had left the cabbage crop and moved to overwintering sites.

Experiment 5: Effect of duration of acclimation at 0 °C on SCP of pre-winter beetles

Pre-winter beetles, collected in mid-August were acclimated for up to 33 days at 0 °C. Samples of beetles were removed at intervals of 1–8 days during this period and their SCPs determined.

Data analysis

Analysis of variance (two-way ANOVA) was used to assess the effect of food plant and starvation on SCP. Mean differences between SCP values were compared using the ANOVA Tukey test. A t-test was used to compare the effect of starvation at low and high temperatures on SCP in overwintered beetles. Temperature-dependent mortality after 1 min exposure to different low temperatures was analyzed with one-way ANOVA. LLT_{50} was calculated by Probit analysis. All analyses were performed with SAS/STAT version 9.1 (SAS Institute, Cary, NC, USA).

Results

Experiment 1: Effect of food plant and starvation on SCP of overwintered beetles

Food plant and starvation both affected the SCPs of overwintered beetles (Table 1). The SCPs of non-starved beetles varied with the crop (food plant) from which they had been collected (Table 1). Those from white cabbage and oilseed rape had similar values (P>0.05) as did those from white mustard and Indian mustard (P>0.05); the difference between these two groups was significant (Table 1). The SCPs of starved beetles were lower than those of non-starved beetles, but there were no significant differences in SCP values of starved beetles collected from different food plants (Table 1). The interaction effect of food plant and starvation was significant but weak (Table 2).

Table 1 Mean (± SE) supercooling points (SCP) of overwintered flea beetles (*Phyllotreta undulata*) collected from four crops and either starved or not for 96 h at 22 °C; n represents sample size

Food plant	n	Non-starved SCP (°C)	n	Starved SCP (°C)
White mustard	10	−9.6 ± 0.8a	10	−16.9 ± 0.7
Indian mustard	10	−10.9 ± 0.7a	10	−18.0 ± 0.5
White cabbage	15	−14.5 ± 1.1b	15	−17.8 ± 0.7
Oilseed rape	16	−13.5 ± 0.5b	15	−17.1 ± 0.4

Different letters within a column indicate significant differences among means (Tukey test: P<0.05).

Experiment 2: Effect of duration of acclimation at different temperatures on SCP of starved overwintered beetles

The SCPs of starved overwintered beetles acclimated at 22 or 0 °C, and measured after 0–8 days, are presented in Table 3. At the start of the experiment (day 0), SCP was identical in both temperature groups, but thereafter decreased in both groups. On day 1, there was no significant difference in SCP between groups but thereafter the rate of decrease was more rapid in beetles kept at 22 °C than in those kept at 0 °C. Thus, on days 2 and 3, SCPs of beetles at 22 °C were significantly lower than those of beetles kept at 0 °C, whereas by day 8, SCP of beetles at 22 °C was significantly higher than that of beetles kept at 0 °C. SCP values were not determined

Table 2 Two-way ANOVA on the effect of food plant and starvation on the supercooling points of overwintered *Phyllotreta undulata* beetles

Effect	d.f.	SS	F	P
Food plant	3	111.91	5.86	0.001
Starvation	1	654.75	102.87	<0.001
Food plant*starvation	3	90.98	4.77	0.004
Error	92	585.58		

Table 3 Mean (± SE) change in supercooling points (SCP) of starved *Phyllotreta undulata* beetles kept at 0 or 22 °C over 8 days. P-values in bold indicate significant differences between temperature groups (t-test: P<0.05); n represents sample size

Day	n	SCP 0 °C	n	SCP 22 °C	t	P
0	16	−13.5 ± 0.53	16	−13.5 ± 0.53	0.00	1.000
1	11	−15.5 ± 0.79	21	−15.3 ± 0.52	0.28	0.779
2	11	−15.0 ± 0.66	21	−16.9 ± 0.37	2.81	**0.011**
3	13	−15.0 ± 0.59	15	−16.8 ± 0.35	2.70	**0.016**
7	10	−16.5 ± 0.22	15	−16.7 ± 0.35	0.36	0.690
8	10	−19.1 ± 0.23	16	−16.8 ± 0.45	3.89	**0.001**

beyond day 8 because most of the beetles kept at 22 °C had died by day 9.

Experiment 3: Effect of sub-zero temperatures on SCP and mortality of non-acclimated and acclimated overwintered beetles

The effect of sub-zero temperatures on the cold tolerance of beetles with and without prior acclimation at 0 °C for 7 days are presented in Figure 1. There was no mortality in either group at −4 °C and, in both, mortality was low at −6 °C. Mortality increased gradually with decrease in exposure temperature (one-way ANOVA: $F_{6,21}$ = 149.247 and 396.069, both P<0.001, in non-acclimated and acclimated beetles, respectively). All non-acclimated beetles died at −18 °C, a few acclimated beetles (2.4%) survived this temperature. Acclimation affected survival at most of the sub-zero temperatures tested. There were significant differences in mortality between non-acclimated and acclimated beetles at temperatures from −6 to −16 °C (Table 4 and Figure 1).

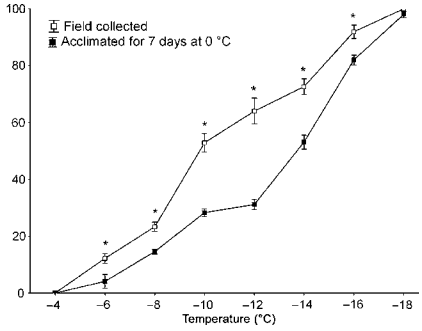

Figure 1 Mean (± SE) mortality of overwintered *Phyllotreta undulata* beetles, after 1 min at various sub-zero temperatures, after being acclimated or not at 0 °C for 7 days. Asterisks indicate significant difference between groups (t-test: P<0.05).

Table 4 Analysis of differences in mortality between acclimated (at 0 °C) or not acclimated *Phyllotreta undulata* beetles after 1 min exposure at various sub-zero temperatures

Temperature (°C)	t	d.f.	P
−6	2.70	6	0.03
−8	4.70	6	0.003
−10	6.90	6	<0.001
−12	6.70	6	<0.001
−14	5.28	6	0.001
−16	3.40	6	<0.001
−18	1.68	6	0.14

Comparisons of the LLT_{50} and mean SCPs for non-acclimated and acclimated beetles are presented in Table 5. Non-acclimated beetles died at temperatures 3 °C higher than their SCPs. Acclimation at 0 °C for 7 days affected both. Acclimated beetles had a mean SCP and a LLT_{50} 3 °C lower than non-acclimated beetles. Thus, the proportional difference between mean SCPs and LLT_{50} remains the same in both groups, whether they were acclimated or non-acclimated to low temperature.

Experiment 4: Effect of collection date on SCP of pre-winter beetles

As the season progressed, SCP values of pre-winter field-collected beetles gradually decreased from −14.5 °C on 15 August to −18.5 °C on 14 September ($F_{4,50}$ = 3.65, P<0.05), as did the variability of SCP of beetles collected on each date (Figure 2).

Experiment 5: Effect of duration of acclimation at 0 °C on SCP of pre-winter beetles

The SCP of pre-winter beetles decreased steadily with duration of acclimation ($F_{7,93}$ = 8.35, P<0.001; Figure 3). Although differences in SCP between days 1 and 2 were not significant (P<0.05), those on days 1 and 2 differed significantly from those on days 3–14. The lowest mean SCP (−21.3 °C) occurred after 18 days of acclimation. Thereafter, further extension of the acclimation period did not decrease the SCPs; there were no significant differences between the SCP of beetles kept at 0 °C for 18, 25, or 33 days.

Discussion

This study has shown that, in the flea beetle *P. undulata*, cold hardiness, as determined by the SCP, is affected by

Table 5 Mean (± SE) supercooling points (SCP) and lower lethal temperatures (LLT_{50}) of overwintered *Phyllotreta undulata* beetles after no acclimation and acclimation at 0 °C for 7 days

Treatment	SCP (°C)[1]	LLT_{50} (°C)	95% fiducial limits	Wald χ^2
Non-acclimated	−13.5 ± 0.53	−10.6 ± 0.16	−11.02–10.20	$\chi^2 = 5.25$, d.f. = 1, P = 0.022
Acclimated	−16.5 ± 0.22	−13.0 ± 0.23	−13.40–12.55	

[1]SCPs came from Table 3.

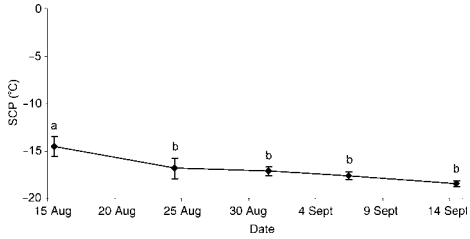

Figure 2 Mean (± SE) supercooling points (SCP) of pre-winter *Phyllotreta undulata* beetles field-collected on various dates. Different letters indicate significant differences between SCPs (Tukey test: P<0.05).

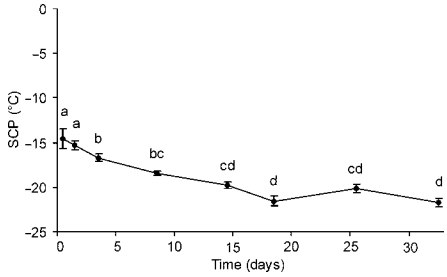

Figure 3 Mean (± SE) effect of duration of acclimation at 0 °C on supercooling point (SCP) of pre-winter beetles of *Phyllotreta undulata*. Different letters indicate significant differences between SCP values for beetles sampled on various dates (Tukey test: P<0.05).

food plant, starvation, and acclimation, and changes as the season progresses; mean SCP varied from −9.6 to −21.3 °C under different conditions. After exposure to sub-zero temperatures, the LLT_{50} and SCP were correlated although the former was a few degrees higher than the latter.

Experiment 1 showed that both food plant and starvation affected SCP in overwintered beetles. The SCP values of field-collected beetles from white cabbage and oilseed rape had lower SCPs than those collected from white and Indian mustard. These differences probably resulted from differences in the digestive properties of the plant material ingested. This is supported by the finding that, after a 4-day period of starvation at room temperature, during which the beetles voided their gut contents (as evidenced by faecal pellets in their vials), no significant differences were found in the SCPs of beetles collected from different crops. Studies of other insects have similarly shown that gut contents can influence their SCPs (Bale, 1980; Sømme & Block, 1982; Lee et al., 1996; Worland & Lukešová, 2000; Suh et al., 2002; Worland, 2005). Nucleation in a supercooled insect usually occurs in the gut content; less cold-hardy food in the digestive tract freezes at a higher temperature than other body parts (Salt, 1953, 1968). For example, SCPs of springtails varied with ingestion of different algae (Sømme & Block, 1982).

Experiment 2 showed that both the duration and the temperature of acclimation affected SCP in overwintered beetles. The SCP of starved beetles declined rapidly from −13.5 to −16.9 °C over the 1st 2 days at 22 °C and then remained at this level for the following 6 days, whereas the SCP of beetles kept at 0 °C declined more slowly over 7 days to −16.5 °C, but reached the lower

value of −19.1 °C on day 8. The more rapid response at the higher temperature was probably the result of increased metabolism with faster dehydration and gut emptying. These results accord with current knowledge of factors affecting cold hardiness in insects and their interactions. Starvation and acclimation to cold are recognised as important factors affecting the SCP (Sømme, 1999; Block, 2002), although their effects may be difficult to distinguish (Worland, 2005). Further, cold and dehydration are not necessarily conflicting evils for the insect as the physiological and biochemical responses to these stresses are similar and both aid survival during overwintering (Block, 1996). Cold and dehydration induce the production of cryoprotectants that lower the SCP, but this takes time. Thus in experiment 2 the SCP of beetles kept at 0 °C declined slowly to reach its lowest value after 8 days.

Experiment 3 showed that field-collected overwintered beetles acclimated at 0 °C for a week tolerated sub-zero temperatures better than non-acclimated beetles; more of the former survived 1 min exposure to sub-zero temperatures ranging from −8 to −16 °C and a few even survived −18 °C. Experiment 3 also showed that the acclimated beetles had a lower SCP as well as a lower LLT_{50} than beetles that had not been so acclimated. For some insects, the SCP is a good indicator of cold hardiness when mortality is assessed after 1 min exposure to sub-zero temperatures (Hodkova & Hodek, 1997). Freeze-intolerant insects tend to have a high supercooling capacity. Their SCPs approximate their lower lethal temperatures; they die when exposed to temperatures at or below their SCP as they do not survive ice formation in their body fluids or tissues (Lee, 1991; Block, 1995; Bale, 1996; Sinclair, 1999). *Phyllotreta undulata* with its high supercooling capacity, down to at least −21.3 °C, appeared to be in this category as no beetles survived temperatures below their SCP. In other insects, the SCP is not such a good indicator of cold hardiness. For example, in aphids, the difference between LLT_{50} and mean SCP can be large, up to 20 °C, so their cold hardiness is not related to their supercooling capacity (Bale, 1996).

In Experiment 3, some pre-freeze mortality of beetles occurred; the LLT_{50}s of both acclimated and non-acclimated beetles were 3 °C higher than their respective SCPs. Mortality after 1 min exposure was greater in both acclimated and non-accliamated beetles than their SCPs would suggest. Some beetles died at −6 °C, considerable above the SCP of both acclimated (−16.5 °C) and non-acclimated beetles (−13.5 °C). Thus acclimation decreased SCP values of beetles, increasing their cold hardiness but the differences between mean SCP and LLT_{50} remained unchanged, ca. 3 °C (Table 3). Thus, in *P. undulata* the occurrence of some pre-freeze mortality indicates that SCP is not the best indicator of cold hardiness in this species. However, because the difference between SCP and LLT_{50} is small and constant there is a good correlation between SCP and cold hardiness. According to Turnock & Fields (2005), complete absence of non-freezing mortality of overwintering insects is probably rare.

Experiment 4 showed that, under field conditions, the SCP values of beetles change over a season. During the 5 weeks (mid-August to mid-September) before they sought overwintering sites, the mean SCP values of pre-winter beetles decreased by 4.0 °C. Variability in SCP values also decreased over these weeks. The greater variability in SCP values during August may reflect the variation in emergence dates of the new generation adults in the sample and differences in the amount of food in their guts. By September, many of the beetles had stopped feeding and had started to migrate from the cabbage field to their overwintering sites, resulting in less variation in SCP of sampled beetles. We were unable to determine the precise period of time needed to achieve the seasonal minimum SCPs under field conditions, because the trend towards lower SCPs was still ongoing in mid-September when the beetles abandoned the field and sampling ceased. The SCP of other insect species is known to decrease slowly toward the autumn (Bale, 1980; Worland, 2005). Cooler temperatures and shortening days encourage natural acclimation, helping to enhance their cold hardiness and potential to survive unfavourable winter conditions.

Experiment 5 showed that the SCP values of field-collected pre-winter *P. undulata* also decreased gradually when acclimated at 0 °C in the laboratory. The decrease was greater than that measured in the field (6.4 compared with 4.0 °C). Further, the lowest SCP value of −21.3 °C, attained after 18 days of acclimation, was lower than that measured in beetles acclimated in the field (experiment 4). Acclimation conditions in the field (experiment 4) and laboratory (experiment 5) differed not only in their temperature regimes (higher and fluctuating temperature in the field vs. constant low temperature in the laboratory), but also in the gut content of the beetles tested. Beetles acclimated in the field may still have been feeding whereas those acclimated in the laboratory were starved. After moving to overwintering sites beetles stop feeding, enabling them to increase their supercooling capacity. The beetles left the field long before the onset of temperatures of 0 °C and below and therefore acclimation and lowering of SCP probably continued in overwintering sites. A long acclimation

period is known to increase cold hardiness in other species too (McDonald et al., 1997).

Cold hardiness of overwintered beetles may not be representative of cold hardiness in new-generation pre-winter beetles. Cold tolerance of beetles that have survived a long winter may either have been reduced or enhanced; less tolerant beetles probably died during the winter leaving a spring population of the more cold-hardy beetles. This remains to be determined.

The abundance of *P. undulata* on cruciferous crops in Estonia indicates that this species is well-adapted to unstable local weather conditions (Hiiesaar et al., 2004). The beetles overwinter near the soil surface under debris, where the minimum temperatures (Kuusik, 1978) are usually higher than their SCP and LLT_{50}. Consequently, overwintering mortality due to low temperature may be rare, for example, only in extremely severe snow-less winters. With the apparent trend towards less severe winters in northern Europe, low winter temperatures may not be a reliable mortality factor in the control of *P. undulata* populations in Estonia.

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Supercooling ability and cold hardiness of the pollen beetle *Meligethes aeneus*

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Key words: seasonality, supercooling point, acclimation, cold tolerance, cold injury, survival, Coleoptera, Nitidulidae

Abstract

Supercooling point (SCP) and cold-hardiness of the pollen beetle *Meligethes aeneus* (Fabricius) (Coleoptera: Nitidulidae) were investigated. Mature eggs from the oviduct were supercooled on average to −28.0 °C and from oilseed rape buds to −24.4 °C; first instars were supercooled to −21.0 °C and second instars to −16.8 °C. Despite their high supercooling ability, none of the eggs survived 24 h exposure to −2.5 °C. The supercooling ability of adults varied significantly among feeding and non-feeding beetles: high SCPs prevailed during the whole warm period, being about −12 °C; low values of SCP of −20 °C dominated in non-feeding beetles. In spring and autumn, beetles displayed the same acclimation efficiency: after 1 week of exposure at 2.0 °C with no access to food their SCPs were depressed equally by about 3 °C. *Meligethes aeneus* beetles have a different response to low temperatures depending on the season. The lowest tolerance was found in reproductively active beetles after emergence from overwintering sites; the time needed to kill 50% of individuals ($Ltime_{50}$) was 56.2 h at −7 °C and the lower lethal temperature needed to kill 50% ($Ltemp_{50}$) after 24 h exposure was −8.6 °C. Cold hardiness increased from midsummer to midwinter; $Ltime_{50}$ was 80 h in August, 182.8 h in September, and 418.1 h in January. Lethal temperature after 24 h exposure was −9.1 °C in August and −9.8 °C in September. In February, after diapause, the beetles started to loose their cold tolerance, and $Ltemp_{50}$ was slightly increased to −9.5 °C. Hibernating beetles tolerated long exposure at −7 °C well, but mortality was high after short exposure if the temperature dropped below −9 °C for 24 h. Despite the season, the beetles died at temperatures well above their mean SCP; consequently, SCP is not a suitable index for cold hardiness of *M. aeneus*.

Introduction

Meligethes aeneus (Fabricius) (Coleoptera: Nitidulidae) is a univoltine species which causes serious damage to cruciferous crops and many other cruciferous plant species (Williams, 2010); the beetles are most successful on oilseed rape, *Brassica napus* L. (Brassicaceae) (Ekbom & Popov, 2004). In spring, the adults start to emerge from hibernation when average temperatures reach 7–8 °C and feed on a diversity of spring flowers until the ovaries of the females attain maturity (Ruther & Thiemann, 1997). Oviposition is restricted to plants of the family *Brassicaceae*. Eggs are laid in the buds and the larvae feed on pollen; the reproductive period may last for 2 months (Ekbom, 1998). Beetles of the new generation emerge in late July or early August and feed on autumn flowers of various plant families for some weeks to accumulate reserves for hibernation. These beetles enter diapause without reproduction.

Before hibernating, most adults migrate to forests, brush, or hedges where they are more insulated from the temperature extremes of open fields. Optimal sites for overwintering are found in the forest areas bordering fields and only few individuals overwinter in the field (Hokkanen, 1993); according to Marczali & Nádasy (2006), the beetles prefer oak forests. Northern regions like Estonia have few oak forests and fields are mostly within bog, scrub, and forest habitats with greater snow accumulation

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and prolonged or rapid fluctuation of low temperatures. Adults spend the winter in obligate (reproductive) diapause which is broken only after a certain cold period, usually before the end of January (Marczali & Nádasy, 2006).

The life history of *M. aeneus* has been extensively investigated (Williams, 2010). Studies include mortality factors of the larvae and adults (Büchi & Nuss, 2000; Büchi, 2002; Hokkanen, 2008) and data about hibernation of beetles under field conditions in both northern (Hokkanen, 1993; Lehrman, 2007) and southern regions of Europe (Marczali & Nádasy, 2006). According to Hokkanen (1993), mortality of hibernating pollen beetles in Finland may reach 85–98%. Lehrman (2007) found only 2.4% winter survival in 2006 in Sweden. Adults, embryos, and larvae may all encounter low temperatures, because, in northern regions, temperatures around zero with night frosts are common during the growing season.

The supercooling point (SCP) is often used to evaluate an insect's cold hardiness as it is a necessary part of winter survival, although mortality may occur at temperatures above the SCP because of chill injuries (Lee, 1991). The SCPs may be used as a comparative cold tolerance indicator in different physiological stages of an insect (Hodkova & Hodek, 2004). Cold hardiness is a common strategy of insects to survive cold periods and it is influenced by many environmental factors like food, temperature, and humidity (Sømme, 1999). In northern regions with unstable winters, cold hardiness is an inevitable prerequisite for winter survival. Cold hardiness is determined as the ability to survive exposure for longer periods at low temperature. In many species, mortality increases with prolonged exposure to subzero temperatures or with decrease in temperature (Block, 1995; Sømme, 1999). However, the length of exposure has been considered the most important factor determining mortality because extremely low temperatures are usually softened by snow cover (Bale, 1991). Prolonged exposure at subzero temperatures may cause injury and mortality even if the insect does not freeze (Renault et al., 2002). In Estonia, overwintering *M. aeneus* beetles may encounter very severe conditions as almost every year there are some short periods with extremely low temperatures. However, despite the severity of the winter, the number of beetles remains continuously high from year to year. Thus, at least part of the *M. aeneus* population must be adapted to survive the range of minimum temperatures it encounters in the microhabitat it chooses. Perhaps there are especially good microhabitats where the beetles can survive in large numbers.

We investigated the cold hardiness and supercooling ability of *M. aeneus*. More specifically, we assessed (1) the SCP of eggs and larvae, (2) seasonal changes in SCP of adults, (3) the influence of acclimation on SCP overwintered and pre-winter beetles, (4) lethal time after exposure of beetles from various seasonal groups to constant subzero temperatures, and (5) lethal temperatures after exposure of beetles from various seasonal groups to different subzero temperature for a constant period of time.

Materials and methods

Insect material

The experiments were performed in 2008, 2009, and 2010. Overwintered *M. aeneus* were gathered from dandelions [*Taraxacum officinale* F.H. Wigg (Asteraceae)] and winter oilseed rape buds and flowers in May and June near Tartu (Estonia, 58°18′N, 26°41′E) New generation beetles were collected in August and September from flowering milkweed [*Sonchus arvensis* L. (Asteraceae)], Canada thistle [*Carsium arvense* (L.) Scop. (Asteraceae)], and nasturtium [*Tropaeolum majus* L. (Tropaeolaceae)]. As *M. aeneus* is difficult to find in its overwintering sites, some beetles were placed in 1-l glass jars, filled to one-third with lightly moistened peat covered with crinkled paper where the beetles could hide. The jars were held in a freezer at +2 to +4 °C until use. Eggs were obtained from the buds of winter oilseed rape (their precise age was unknown) or removed from the oviducts of females before morphogenesis. Larvae were obtained from the flowers of winter oilseed rape; their instar was determined by head capsule diameter (first instar = 0.19 mm, second instar = 0.3 mm).

Supercooling points

The SCPs of *M. aeneus* eggs, larvae, and adults were measured by using a copper-constantan thermocouples-thermometer (RS-232 Data logger Thermometer; TES Electrical Electronic, Taipei, Taiwan). The low temperatures were attained by deep-freeze HF-103 (−30 °C). To obtain field-collected beetles with empty guts, they were held at room temperature for 24 h with no access to food before starting measurements. Prior to supercooling, the beetles were anaesthetized by low temperature (0 to −2 °C) to render them immobile and then fixed individually by a thin Vaseline layer to the top of the thermocouple and sealed in a plastic tube. Tubes were placed in a cotton-lined container and transferred into the freezing chamber. A cooling rate of 1 °C min^{-1} was used. The temperature at which freezing produced a release of latent heat was taken as the SCP of the individual. The numbers of eggs, larvae, and beetles are presented in Figures 1 and 2.

Acclimation efficiency

To examine the effect of cold acclimation on the supercooling ability of overwintered spring and pre-winter

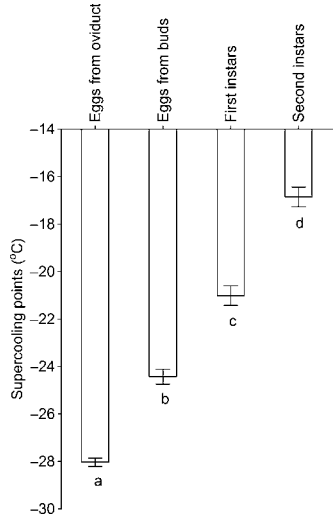

Figure 1 Mean (± SE) supercooling points of preimaginal stages of *Meligethes aeneus*. Means with different letters are significantly different (Fisher LSD test: P<0.05). Both groups of eggs: n = 15; both groups of larvae: n = 20.

autumn population, beetles collected on 6 June and 3 September were exposed to 2 °C for 1 week in darkness with no access to food. SCPs were measured before and after acclimation (n = 17 for each group of beetles).

Cold-tolerance

To assess the cold tolerance of eggs, they were removed from buds and placed on moist filter paper in Petri dishes and transferred for 24 h to one of three low temperatures, −2.5, 0, and +2.5 °C (n = 20, 28, and 27, respectively). After exposure, the eggs were incubated at room temperature and their survival rate was assessed by hatchability. As a control, eggs removed from buds were held continuously at 22 °C (n = 15).

The effect of long-term exposure to constant low temperatures on mortality of beetles ('quantity factor'), and the ability to withstand extreme low temperatures ('intensity factor') [Payne (1926) in Renault et al. (2002)] was assessed after emergence of beetles from overwintering places (early June), after emergence of a new generation beetles from the soil (early August), before the beetles left for their overwintering sites (September), when the beetles were in deep diapause (mid January; quantity factor), or after termination of diapause (late February; intensity factor).

In the first experiment, the survival of beetles was determined at constant −7 °C. This temperature was selected because it was higher than the beetle's mean SCP and a temperature the beetles were likely to encounter in their overwintering sites under field conditions. Batches of beetles were wrapped in glass vials lined with filter paper and transferred into the thermostat for various periods of time: 24, 48, 72, 96, 120, 144, and 312 h. One batch per treatment was removed at daily intervals. In the second experiment, the beetles were exposed for a constant period of time (24 h) to various subzero temperatures: −7, −9, −11, −13, −15, and −17 °C.

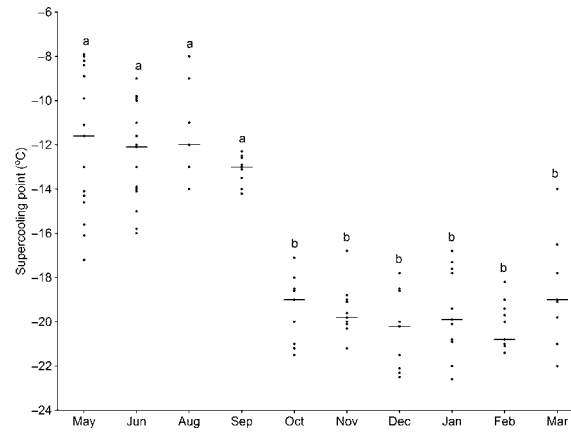

Figure 2 Seasonal changes of supercooling points in adults of *Meligethes aeneus*. Horizontal lines indicate median values, the dots are the raw data. Medians capped with different letters are significantly different (Tukey HSD test for unequal n: P<0.05). Each feeding group (May-September): n = 17; each non-feeding group (October–March): n = 11.

The beetles were cooled at a rate of 1 °C h^{-1} down to the required temperature, followed by a warming to room temperature at the same rate. The temperature fluctuation inside the vials did not exceed 0.5 °C. Constant cooling and warming rates were achieved using a liquid thermostat (Ministat 230w-2; Huber, Offenburg, Germany). Following exposure, the beetles were kept in Petri dishes at room temperature. Low temperature effect was scored twice: development of chill (cryo) injuries and mortality was assessed 2 h after transfer of beetles from cold to room temperature. Based on the method described by Koštal et al. (2007), the beetles were considered fit if they were active and able to move in a normal manner. Beetles were considered injured if they fell over and could move only short distances. Beetles not responding to tactile stimulation were considered dead. The second assessment was made after 24 h (sometimes 48 h) by which time the beetles had either recovered from their injuries or were obviously dead.

Sex and supercooling points

The sex of beetles was determined by the presence or absence of an aedeagus (male genitalia). In the preliminary test, the SCPs of 40 randomly-chosen overwintered feeding beetles were measured and their sex determined. Because sex did not affect their SCP, mixed sex beetle groups were thereafter used in all experiments.

Statistical analysis

Differences between SCP of different preimaginal stages were analysed by t-test and one-way ANOVA. SCPs of adults of overwintered and pre-winter beetles prior to and after acclimation was compared by t-test. Seasonal changes in SCP were analysed with Tukey's honestly significant difference (HSD) test for unequal sample sizes. Differences in the survival rate of beetles between seasonal groups depending on exposure time or temperature were compared by two-way ANOVA. In addition, lethal time to reach 50% mortality of beetles ($Ltime_{50}$) after exposure to constant −7 °C, and lower lethal temperature at which 50% of beetles died ($Ltemp_{50}$) when exposed for constant 24 h were calculated by probit analyses (Finney, 1962).

Results

Supercooling point and cold tolerance of preimaginal stages

The preimaginal stages of *M. aeneus* varied in their supercooling ability (Figure 1). The difference in the SCP of eggs and larvae was significant ($t = 12.52$, d.f. = 68, $P<0.001$) as was that of their ages ($F_{3,66} = 162.0$, $P<0.001$). Mature eggs from the oviduct had the lowest SCP value; they supercooled on average to −28 °C (ranging from −26 to −29 °C). The mean SCP of developing eggs from buds was significantly higher (−24.4 °C) and with greater individual variability (−21 to −26 °C). The supercooling ability of larvae was lower, first instars freezing at −21 °C (−17 to −23 °C), second instars at −16.8 °C (−11 to −20 °C).

Low temperatures above 0 °C did not inhibit embryonic development, all eggs survived and normal larvae hatched after exposure to 2.5 °C. When the temperature dropped to 0 °C, 89.3% of the eggs survived whereas at −2.5 °C none of the eggs survived. Manipulation of eggs during their removal from the buds did not damage them as all hatched in the control group kept at a constant 22.0 °C.

Seasonal changes in supercooling point of adults

Mean SCP varied significantly among feeding and non-feeding beetles (one-way ANOVA: $F_{9,121} = 47.848$, $P<0.0001$). High SCPs prevailed in the warm part of the season (Figure 2). The mean supercooling ability of feeding beetles with mean values of about −12.0 °C remained almost the same from May to September: there was no difference in the SCPs of overwintered beetles during the maturation feeding period in spring, reproductive activity period in summer, or in beetles of the new generation during the reserve accumulation period in autumn. In October, when the beetles stopped feeding, their SCP decreased sharply and stabilized nearly on the same level for the whole cold period. A small but statistically non-significant depression of SCP was observed from October to December and a small increase from February to March (the last assessment). The lowest individual SCP (−22.6 °C) was measured in January and the highest (−7.6 °C) in May. Individual variation in the SCP value for overwintering beetles was from −14 to −22.6 °C and for feeding beetles from −7.6 to −17 °C.

The influence of acclimation on supercooling point of overwintered and prewinter beetles

The overwintered reproductively active beetles collected in spring and the new generation beetles collected in autumn, before they stopped feeding, had almost the same mean SCPs, −13.9 and −13.2 °C, respectively ($t = 0.98$, d.f. = 30, $P = 0.33$). After acclimation, the mean SCPs of both generations were depressed to the same extent, about 3, −16.4 and −16.3 °C, respectively ($t = 0.03$, d.f. = 30, $P = 0.97$). Differences between the SCP of beetles before and after acclimation were significant ($t = 4.64$, d.f. = 62, $P<0.001$).

Lethal time

Figure 3 illustrates the development of cold injuries and mortality of *M. aeneus* beetles from various seasonal

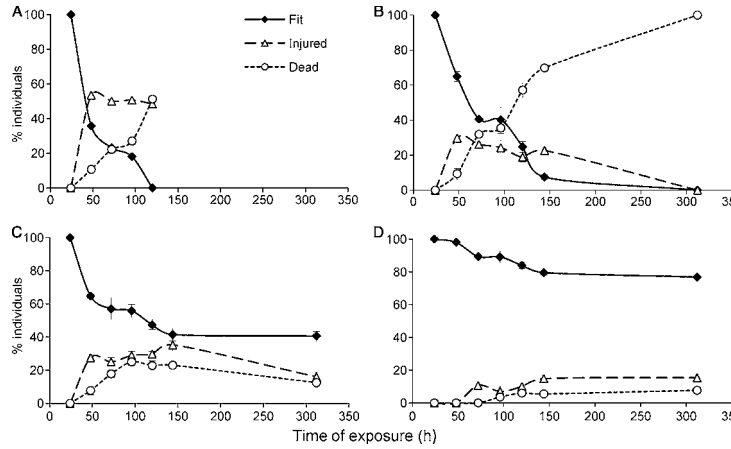

Figure 3 Development of chill injury and mortality in *Meligethes aeneus* beetles exposed to −7 °C for different periods of time. Beetles were from four seasonal groups: (A) May, (B) August, (C) September, and (D) January. Assessment was made 2 h after the end of cold exposure. Sample sizes are given in Table 1. Mean values are indicated (± SE).

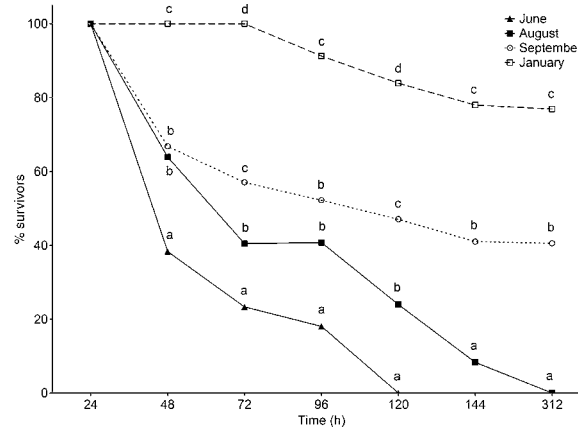

Figure 4 Mean (± SE) survival of *Meligethes aeneus* beetles in various seasonal groups after exposure to −7.0 °C for different periods of time (quantity factor). Assessment was made 24 or 48 h after the end of cold exposure. Different letters above the means at the same exposure time indicate significant differences between seasonal groups (Fisher LSD test: $P<0.05$).

groups after exposure to −7 °C for different periods of time (estimated 2 h after the end of exposure and re-warming at room temperature). Survival of these beetles 24 or 48 h after the end of exposure is shown in Figure 4.

Data of two-way ANOVA analysis show that season ($F_{3,83} = 631.99$, $P<0.0001$) as well as exposure time ($F_{6,83} = 283.08$, $P<0.0001$) had a significant effect on the survival of beetles. There was significant interaction between seasonal groups and exposure time ($F_{18,83} = 22.52$, $P<0.0001$). The longer the exposure, the greater the mortality in all groups. Probit analysis data presented in Table 1 show that the time needed to cause 50% mortality of beetles ($Ltime_{50}$) at −7 °C varied significantly with season.

Irrespective of season, beetles survived 24 h exposure at −7 °C well. The lowest cold tolerance was found in

Table 1 Mean (± SE) lethal time [$Ltime_{50}$ (h); time required for 50% mortality at constant -7 °C] and lethal temperature [$Ltemp_{50}$ (°C); temperature required for 50% mortality after 24 h exposure] of *Meligethes aeneus* beetles

Ranking time	n	$Ltime_{50}$	95% fiducial limits	n	$Ltemp_{50}$	95% fiducial limits
June	333	56.2 ± 0.4a	44.3–65.6	231	−8.6 ± 0.2a	−8.7 to −8.0
August	575	80.0 ± 0.2b	73.0–86.4	294	−9.1 ± 0.9b	−9.3 to −8.8
September	406	182.8 ± 0.1c	120.9–347.8	587	−9.8 ± 1.1b	−10.5 to −8.9
January/February	376	418.1 ± 0.2c[1]	336.0–597.5	374	−9.5 ± 0.7b[2]	−10.3 to −8.8

Different letters within a column indicate significant differences between seasonal groups based on the lack of overlap of their 95% fiducial limits.
[1]Determined in January.
[2]Determined in February.

overwintered reproductively active beetles in June. After 48 h exposure, over 50% of them showed chill injuries (Figure 3A) and about 10% died; only a few of the injured beetles in this group recovered during the following 48 h at room temperature. None of the June beetles remained fit after 96 h exposure; they were either cold injured (49%) or dead (51%). The time required to cause 50% mortality of the beetles ($Ltime_{50}$) was 56.2 h.

The cold hardiness of the new generation in August was higher as the beetles tolerated longer exposure at −7 °C than spring beetles. After 48 h exposure, about 60% of the beetles survived and about 30% showed chill injuries, from which none of the injured beetles later recovered. After 72 and 96 h exposure, the proportions of live, dead, and injured beetles were almost equal (Figure 3B). Extension of the exposure time to 120 h resulted in over half the beetles dying with the proportion of fit and injured ones being almost equal. After 144 h exposure, only a few beetles survived, and after 320 h, all died. $Ltime_{50}$ was 80 h.

At the end of September, when the beetles had almost completed their pre-winter reserve accumulation feeding period, their cold tolerance was distinctly increased. However, we did not find significant differences in survival in comparison with the mid-summer group after 48 and 96 h exposure, but after 120, 144, and 312 h mortality was significantly lower in this group. The proportion of dead beetles was slightly greater than that of injured ones. $Ltime_{50}$ was extended to 182.8 h.

The peak of cold tolerance was recorded in mid-January. After 48 and 72 h all the beetles survived. Some dead and injured beetles were found after 96 h exposure. Nearly 77% of beetles survived 312 h exposure. The calculated $Ltime_{50}$ increased to 418.1 h.

Lethal temperature

Figure 5 illustrates the responses of *M. aeneus* beetles from various seasonal groups following 24 h exposure to different low temperatures assessed 2 h after the end of exposure. The survival of these beetles 24 or 48 h after the end of cooling is presented in Figure 6. Probit analysis data, where lower lethal temperature at which 50% of beetles died ($Ltemp_{50}$) when exposed to constant time 24 h, are shown in Table 1.

At −9 °C, chill injuries and mortality of summer beetles developed more or less equally; in the autumn group, the proportion of beetles with cold injuries was greater than the proportion of beetles that died; in overwintered beetles the reverse occurred. Temperatures of −11 and −13 °C caused mortality of most summer and overwintering beetles, few showed cold injuries; in the autumn group the proportion of beetles with cold injuries dominated overwhelmingly. After exposure to −15 °C, only a few beetles showed chill injuries, most of them were dead.

Two-way ANOVA shows that season ($F_{3,59} = 117.15$, $P<0.0001$) and temperature ($F_{5,59} = 3569{,}74$ $P<0.0001$) had a significant effect on survival but that survival varied among seasonal groups (interaction: $F_{15,59} = 67{,}79$, $P<0.0001$). Most vulnerable to cold exposure were overwintered reproductively active beetles in early June, the temperature of −9 °C was lethal for most of them. Most resistant were beetles in late September after termination of feeding, when 70% of them survived exposure to −9.0 °C. Survival of the August group was much greater than in June but lower than in September. At the end of February, after termination of diapause, the beetles started to lose their cold tolerance as significantly fewer beetles survived exposure to −9 °C than in September (Figure 6).

When the temperature dropped to −11.0 °C, beetle survival decreased substantially. None of the spring beetles survived this temperature; there were no significant differences among summer, autumn, or winter beetles with about 10% survival. Few autumnal and overwintered beetles survived exposure to −13 °C; only single autumnal beetles survived a temperature of −15 °C and all died at −17 °C.

Lethal temperature causing 50% mortality in the *M. aeneus* population, showed a significant difference only with overwintered spring beetles; there were no significant

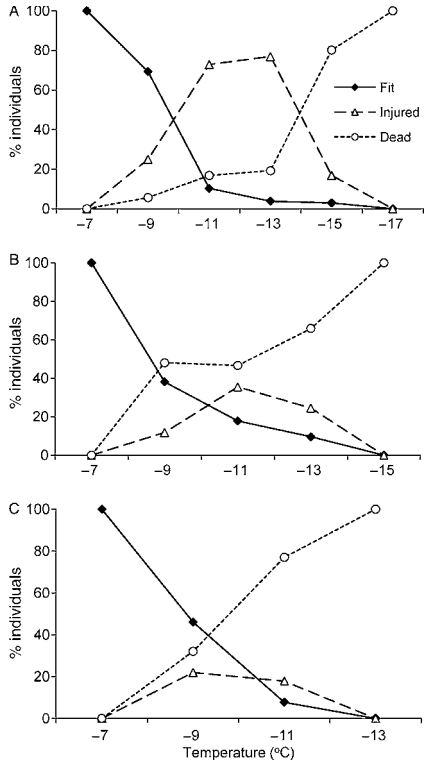

Figure 5 Development of chill injury and mortality in *Meligethes aeneus* beetles after 24 h of exposure at different subzero temperatures. Assessment was made 2 h after the end of cold exposure and recovery of beetles at room temperature. Beetles were from three seasonal groups: (A) August, (B) September, and (C) February.

differences among the other seasonal groups because the 95% fiducial limits overlap (Table 1). A temperature of ca. −9 °C was the valid survival limit for beetles of the new generation after 24 h exposure.

Discussion

Supercooling point and low temperature survival of preimaginal stages

Supercooling ability of *M. aeneus* varied not only by developmental stage but also by age within the same stage. The SCPs of mature eggs removed from the oviduct before morphogenesis had narrow variability and were significantly lower than that of the eggs from oilseed rape buds. Differences in low temperature sensitivity depending on the age of the embryo in other species have been described. Leopold (2000) found that 3-h-old embryos of the house fly (*Musca domestica* L.) were more tolerant to cold storage than 1-h-old embryos. Kishaba & Henneberry (1966) found that the viability of 2-day-old *Trichoplusia ni* (Hübner) was higher than that of 3-day-old eggs. According to Kittlaus (1961), the sensitivity of *Leptinotarsa decemlineata* Say eggs to negative factors is different at different stages of embryogenesis in which it can change within a few hours, depending on the peculiarities of the morphogenesis and the related level of metabolism. We did not know the embryonic stage of the eggs from buds, so the high variability in individual SCPs may have been caused by differences in their age. Although *M. aeneus* eggs had a high supercooling ability with a value much lower than −20 °C, they were very susceptible to subzero temperatures; 24 h of exposure at −2.5 °C caused 100% mortality. The same results were obtained with *Habrobracon hebetor* (Say) eggs and larvae; despite their low SCPs they died at temperatures between −2 and −1 °C (Carrillo et al., 2005). Temperatures above zero but below their developmental threshold of +2.5 °C did not prevent further embryonic development of *M. aeneus* eggs.

Pollen beetle larvae had somewhat lower but nevertheless good supercooling ability. According to Lee (2010), the lack of efficient nucleators in the larval haemolymph and other tissues enables them to supercool extensively. The SCP increased from −21 °C in newly hatched larvae to −17 °C in second instars. We did not manage to identify the real cold tolerance of larvae because they were too vulnerable to manipulative damage; even the larvae of the control group, which did not experience cold stress, ceased development after temporary removal from the buds. Considering that the beetles start to oviposit in June and the eggs and larvae remain protected by buds and flowers, they are at low risk of freezing to death due to low temperature.

Seasonal changes in supercooling point of adults

Seasonal changes in SCP values have been reported by many authors (e.g., Koštal & Šimek, 1995; Koch et al., 2004; Worland, 2005). In our experiments, beetles exhibited significantly higher SCPs in the warm period than in the cold period. We did not find a meaningful difference in the SCP of individuals during the active period, irrespective of their physiological state (mature feeding, reproductive activity period, or pre-winter feeding for accumulation of reserves). Low supercooling ability of

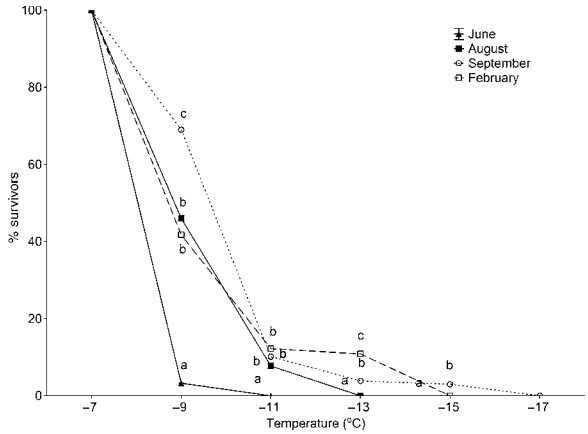

Figure 6 Mean (± SE) survival of *Meligethes aeneus* beetles in various seasonal groups after exposure to different subzero temperatures for 24 h (intensity factor). Assessment was made after 24 or 48 h recovery of beetles at room temperature. Different letters above the means at the same temperature indicate significant differences between seasonal groups (Fisher LSD test: $P<0.05$).

feeding insects is explained by food particles in their alimentary tract which induce ice formation at relatively high temperatures (Carrillo et al., 2005; Zachariassen et al., 2008) and with high water content (Block, 2003). The SCP of *M. aeneus* beetles decreased dramatically from September to October, when the beetles stopped feeding. From October to March, there were no significant changes in supercooling ability and SCP remained at a low level for the whole cold period. The SCP of non-feeding insects is influenced by different factors such as the absence of ice-nucleating components in the gut, dehydration of the organism, and accumulation of antifreezes and cryoprotectants (Lee et al., 1996). An increase in supercooling ability is often connected with diapause (Hodkova & Hodek, 2004) but not inevitably so as diapause may have evolved many times independently (Denlinger, 1991). It seems that diapause is not an obligate prerequisite for the high supercooling ability of *M. aeneus* beetles because diapause terminates at the end of January (Marczali & Nádasy, 2006) and thereafter they remain inactive only by cold quiescence, yet their SCP did not change significantly, remaining at a very low level during February and March.

Good evidence for the high supercooling ability not being associated with diapause is provided by the results of the acclimation test with overwintered and pre-winter *M. aeneus* beetles. After deprivation of food for 1 week at 2 °C, the SCP of both groups of beetles decreased to the same extent. Lee (1991) considers that such elevation of supercooling ability is non-adaptive and is related to the dehydration of the organism and the lack of ice nucleating agents in the gut rather than diapause state. Such an increase in supercooling ability may diminish the risk of mortality in early spring or late autumn because of night frost or rapid decrease of temperatures.

Cold tolerance

Cold tolerance is defined as the ability to survive exposure for long periods to different low temperatures (Sømme, 1996). Earlier studies have shown that survival of insects at low temperatures is time and temperature dependent, mortality rate increases with time of exposure or decrease in temperature (Sømme, 1996), and cold tolerance changes with season (Danks, 1978; Koštal et al., 2001, 2004). Low temperatures, daylight, food, and humidity can be signals for insects to synthesize various cryoprotectants in preparation for overwintering to protect them from cold injury (Block, 1995). Our investigations established that seasonality had a significant influence on low temperature survival after long exposure to moderately low subzero temperature, but the ability to withstand extreme low temperatures ('intensity factor') did not depend so much on season, because $Ltemp_{50}$ after 24 h exposure did not differ significantly between new generation beetles in August, September, or February; differences were found only with the overwintered generation in June. The majority of the new generation beetles survived 24 h exposure at −9 °C which seems to be the lower thermal limit they could tolerate; when the temperature dropped even more, few beetles survived. In contrast, overwintered beetles were very sensitive as almost all of them died already at −9 °C.

The ability to survive long exposure at −7 °C increased gradually from August to January. Many authors associate cold hardiness with diapause, which usually develops at

the same time (Lee, 1991; Koštal & Šimek, 1995; Pullin, 1996; Hodkova & Hodek, 2004). The pollen beetles acquired relatively high cold tolerance already in September before migration to their overwintering sites. Such increase was caused by natural cold acclimation during the autumnal decrease of ambient temperatures and the onset of diapause. The beetles were most cold tolerant in mid-January before the end of diapause with nearly 77% survival after 20 days of exposure at −7 °C.

After termination of diapause in February, only the intensity factor was tested in our experiments and it suggests that, at that time, the beetles started to lose their cold hardiness. Although $Ltemp_{50}$ did not show a statistically significant difference in new-generation beetle survival, none of the beetles survived −15 °C in February contrary to some in September; survival at −9 °C in February was significantly lower than at the end of September (Figure 7). A similar effect was observed for *Anthonomus pomorum* L. whose cold hardiness was higher in December compared to the end of dormancy in March when the beetles had terminated the diapause (Čtvrtečka & Ždárek, 1992).

Cryo-injury

Meligethes aeneus beetles died after exposure to subzero temperatures because of cold injury. Cold injury is distinguished as either chilling or freezing injury (Denlinger & Lee, 1998) and is recognized as a wide-spread cause of death of insects overwintering in supercooled state (Knight et al., 1986; Nedvěd, 2000; Hodkova & Hodek, 2004; Koštal et al., 2004; Renault et al., 2004). The nature of chill injury is very complex because temperature directly affects various physiological processes and biophysical structures simultaneously. The reasons for chill injuries have been discussed extensively (Nedvěd, 1998, 2000; Koštal et al., 2004, 2007; Renault et al., 2004). Temperatures below the developmental threshold but above SCPs might induce injuries in the absence of freezing if the exposure time is sufficiently long; accumulated injuries may be fatal (Nedvěd, 1998). The temperature of −7 °C used in this investigation was above the SCP (−8 to −22 °C) and far below the developmental threshold (+10 °C) of *M. aeneus*. Beetles from different seasonal groups showed chill injuries if the exposure time was increased to at least 48 h, except for the January group where the beetles started to show injuries after 96 h exposure. Injury symptoms refer to damage of the nervous system. Movement of the beetles was uncoordinated because their tarsae with ungues did not hook into the substrate while being bent upwards. Some beetles could only shamble on their prothoracic legs, the other legs were completely paralyzed. According to Kelty et al. (1996), the nerve and muscular system are the most sensitive to chill injury. After short exposure, some chill injuries are reversible when the insects return to higher temperatures (Renault et al., 2004). In our experiment, only a few beetles recovered after 48 h at −7 °C and, if the exposure time was increased, all injured beetles died within 1–2 days. At constant exposure for 24 h, injuries accumulated more quickly at lower temperatures. Cold injuries induced significant mortality in all seasonal groups at −9 °C; if the temperature dropped to −11 and −13 °C the main cause of mortality in the autumn group was chill injury. The high proportion of chill injuries indicated that they are as fatal as freezing.

Supercooling ability in relation to cold tolerance

Low temperature survival is not always connected with changing SCP (Koštal et al., 2001). Increase in cold hardiness of *M. aeneus* beetles corresponds with a decrease in their SCP only in late autumn. After cessation of feeding, the SCP of beetles dropped rapidly and remained low until the spring without considerable fluctuations. However, changes in cold hardiness run differently, following the development of diapause. The ability to survive long exposure to subzero temperatures differed significantly among the physiological states defined by season. Cold tolerance started to increase already in August with the decrease in photoperiod (onset of diapause induction), was enhanced in September during autumnal decrease of ambient temperature (acclimation and further diapause development), and achieved a maximum in mid-January (deep diapause). In February, when development was prevented only by low temperature, the beetles started to lose their cold tolerance, although the SCP of beetles did not change and remained at a low level. Consequently, cold tolerance is not completely associated with the suppression of SCP and the ability to supercool is not connected with the diapause syndrome in *M. aeneus* beetles.

Previous studies have demonstrated that SCP is not a suitable index for cold hardiness of insects because of pre-freeze mortality at temperatures above the SCP (Bale, 1987; Nedvěd, 2000). Low temperature exposure of *M. aeneus* for 24 h indicated that, irrespective of season, the beetles died at temperatures well above their mean SCP. For instance, non-feeding beetles supercooled to about −22 °C, although significant mortality occurred at −11 °C and no beetles survived −17 °C. The mean SCP was also several degrees lower than the $Ltemp_{50}$ of beetles despite the season. These data confirm that SCP is not applicable as an indicator of cold hardiness for *M. aeneus* beetles.

Cold tolerance of overwintering *M. aeneus* beetles suggests that they can survive temperatures they may encounter in cold winters in northern regions. However, mortality of beetles at subzero temperatures in the steady

conditions of the laboratory may not reflect their survival in nature. The high mortality of overwintering beetles established by Hokkanen (1993) and Lehrman (2007) in natural overwintering conditions seems to be the result of several factors, not only low temperatures. Thus, the maintenance of high spring populations of *M. aeneus* in the Estonian climate, irrespective of the harshness of the winter, leads us to conclude that under natural conditions, more beetles survive due to their ability to locate favourable overwintering microhabitats. Additional field experiments are required to identify the real mortality under the conditions of the agricultural mosaic landscapes in Estonia.

Acknowledgements

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Low temperature survival of post-eclosion stages of the potato rot nematode *Ditylenchus destructor* Thorne (Tylenchida: Anguinidae)

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Summary – The potato rot nematode, *Ditylenchus destructor*, may experience extreme environmental stress caused by freezing temperatures when overwintering in the field. A series of laboratory experiments were conducted in order to clarify overwintering strategies of the nematode. The current study aimed to examine the cold tolerance of this species in various aqueous environments as well as in plant tissues in order to determine its ability to survive 24 h exposure to subzero temperatures. Cold tolerance of adults and fourth-stage juveniles was significantly lower than that of younger juveniles. The lower lethal temperature of adults was −15°C. Although external ice formation affects all life stages, a few second-stage juveniles were able to survive temperatures as low as −30°C. The results expand the current understanding of freezing survival in the potato rot nematode and will lead to better comprehension of its ability to withstand subzero temperature conditions.

Keywords – cold tolerance, freezing tolerance, mortality prediction, overwintering.

The potato rot nematode, *Ditylenchus destructor* Thorne, can become important as a pest of potatoes at temperatures of 15-20°C and at a relative humidity above 90% (CAB International, 2010). Andersson (1971) summarises the results of his study by the statement that, even in heavily infested fields, populations of this nematode can be eradicated or decreased if the field is kept free from biennial or perennial host plants for a few years. However, in current agriculture and horticulture, weeds may play a desirable role, providing diversity, ecosystem functions and supporting many other species (Marshall *et al.*, 2003). *Ditylenchus destructor* has a very wide range of host plants and fungi, which is why this pest presents a serious threat in newly cultivated fields with an unrecorded cropping history, especially if reduced tillage is practised. A positive relationship between less intensive soil management practices and the incidence of herbivorous nematodes has been established by several experiments (Thomas, 1977; Hendrix *et al.*, 1986; Hou *et al.*, 2010). Thus, potato rot nematode may become an increasing problem in contemporary agriculture (Svilponis *et al.*, 2008) even though good phytosanitary practices in seed potato production would greatly assist in escaping economic damage.

Since the potato rot nematode may encounter adverse winter conditions in the field in northern temperate areas, it is necessary to study cold tolerance of the pest in detail. Nematodes that survive low temperatures in their natural habitats are cold tolerant (Wharton, 2002). The main model for studying nematode cold tolerance has been an Antarctic nematode, *Panagrolaimus davidi* (Wharton, 2002, 2003), which exhibits atypical survival abilities when compared with other species of nematodes (Smith *et al.*, 2008).

Most of the experimental data on ditylenchid (stem nematode) cold survival date back 40-50 years. Several authors have observed survival of ditylenchids in field conditions. According to Ustinov and Linnik (1955),

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living potato rot nematodes were discovered in spring 1954, even before the planting of potato in fields that were used for potato growing the previous season. A related species, the bulb nematode *D. dipsaci*, survived after overwintering on the surface and at a depth of 10-50 cm in Gorky Oblast, Russia (Kir'yanova, 1951). Without a resistant resting stage, the potato rot nematode overwinters in soil as adults or juveniles and may multiply by feeding on alternative weed hosts and on fungal mycelia. Its survival in soil is enhanced, for example, by the presence of corn mint *Mentha arvensis* L. and unharvested potato tubers. In *D. dipsaci*, the most cold resistant stage has been found to be the fourth-stage juvenile (J4) (Bosher & McKeen, 1954). Thorne (1961) reported that *D. destructor* overwintered in USA field soil as eggs and coiled adults. Ustinov and Tereshchenko (1959) suggested that the winter survival of potato rot nematode is facilitated by egg anhydrobiosis, which makes it capable of enduring low temperatures almost to the point where the soil freezes. *Ditylenchus dipsaci* has been reported to be freeze-susceptible but avoids freezing by supercooling with a mean supercooling point (SCP) of -21.7 ± 0.7°C (Wharton *et al.*, 1984). Stem nematodes remain mobile almost until their freezing point (Shubina, 1985). According to Ladygina (1957), the stem nematodes of potato and onion do not stop moving at 3-5°C, even though they become less active. In infested tubers, bulbs and soil they remain capable of invasion, reproduction and development even after being frozen to -18 to -28°C for 2 days or -11 to -27°C for 14 days; lower temperatures were not tested. Lower lethal temperatures of ditylenchids lie within the negative temperatures below -20 to -28°C (Ladygina, 1957).

Nematodes, as aquatic organisms, are subjected to inoculative freezing when the water in their external environment freezes, unless they are able to prevent it by the presence of protective structures such as eggshell or sheath (Wharton, 1995). The current study aimed to examine the cold tolerance of hatched juveniles and adults (post-eclosion stages) of *D. destructor* in various aqueous environments, as well as in plant tissues. The purpose of the experiments was to establish which media are more favourable for *D. destructor* in ecologically significant sub-zero temperatures, how the population responds to exposure to negative temperatures, and which life stages appear to be most resistant to cold.

Materials and methods

The nematode-infested potato tubers were collected at Viljandi county, Estonia (58°29′N, 25°36′E), in January 2010. The stock material was stored at +4°C for the duration of the experimental series.

The first experiment was set with the purpose of optimising experimental procedures. Hand-picked adults were placed into 1.5 ml Eppendorf tubes containing either 200 μl water, M9 buffer (De Ley & Mundo-Ocampo, 2004) or gravel moistened with water. Additionally, samples of 0.268 ± 0.025 g of infested potato tissue (standard sample) were taken by a cork borer size 1 (internal diam. 3.5 mm). In total, eight replicates were made with water, five with gravel and potato and 11 with M9 buffer. The number of replicates in water and buffer was increased in order to get more reliable results. The samples were cooled and reheated at 1°C min^{-1}. After 24 h cold exposure at -5 ± 1°C in the liquid thermostat (Ministat 230w-2; Huber, Offenburg, Germany; -33 to 200°C), nematodes were removed from the thermostat and kept at +4°C for 24 h to recover before estimation of survival. Mortality was determined by counting the individuals that did not respond to mechanical stimulation by a bristle.

In the second experiment, population survival was assessed. Standard samples of infested potato tissues were transferred into 1.0 ml Eppendorf tubes, which were placed in test tubes into the liquid thermostat and exposed to temperatures of -5, -8, -18 or -30°C for 24 h. Nematodes were counted and the survival of different post-eclosion stages was assessed after the 24 h recovery period. The counts of younger juvenile stages, second- (J2) and third-stage (J3), were combined. The results were compared to that of control samples kept at room temperature (+22°C) for the same period of time. There were five replicates at each temperature.

The probit models with SAS 9.1 (SAS Institute, Cary, NC, USA) procedures PROBIT and GLIMMIX were fitted to estimate and test the effects of media, temperature and age. The discrete effects of media in the first experiment and of age and temperature in the second experiment were compared using suitably defined contrasts. Additionally, the continuous changes in mortality were modelled and the lethal temperatures for 50 and 90% mortality of nematodes were estimated considering the temperature as a continuous factor and applying the probit-regression corrected for control mortality at 22°C using Abbott's formula. A P value < 0.05 was considered statistically significant.

Results

In the first experiment, mortality rates of the potato rot nematodes in different media ranged from 31 to 97% (Table 1). Most favourable for low temperature survival (and statistically not different, $F_{1,25} = 0.03$, $P = 0.86$) were potato and M9 buffer. The mortality in water and gravel was significantly higher than in potato and M9 buffer ($F_{1,25} = 145.90$, $P < 0.001$), but did not differ mutually ($F_{1,25} = 0.96$, $P = 0.34$).

In the second experiment, mortality of the different stages of the potato rot nematode exposed to subzero thermal regimes ranged from 32 to 100% (Table 2). There were no statistically significant differences between age groups at 22°C ($P > 0.05$). Cold tolerance of adults and J4 at −8°C was significantly lower than that of younger juveniles ($F_{1,12} = 77.19$ and $F_{1,12} = 34.75$, respectively, $P < 0.001$). Also the difference between J4 and adults was statistically significant at −8°C ($F_{1,12} = 24.66$, $P < 0.001$). At −18°C there were no significant differences in mortality between adults and J4 ($F_{1,12} = 0.00$, $P = 0.98$), but the cold tolerance of J2 and J3 was still significantly higher ($F_{1,12} = 18.80$, $P < 0.001$). Studying the speed of changes in cold tolerance caused by the decrease of temperature, it appears that adults are the most sensitive and J2 and J3 the most resistant. The mortality of adults increased rapidly and was statistically significant only between temperatures −5°C and −8°C ($F_{1,28} = 162.21$, $P < 0.001$), whilst the mortality of juveniles changed more slowly, showing statistically significant changes until −18°C for J4 and −30°C for J2 and J3 (Table 2). The same differences are visible in Figure 1 where the mortality is modelled on a continuous scale. The variability in mortality is greater among younger nematodes compared with the adults, which react similarly to the temperature changes.

The highest lethal temperature for 90% of population was exhibited by adults (−7.4°C) when compared with J4 and younger juveniles (−9.4°C and −14.5°C, respectively). Adults exhibited 100% mortality at temperatures below −15°C. Although external ice formation affects all life stages, a few J2 were able to survive temperatures as low as −30°C.

Discussion

This study shows that the lethal temperature at which 90% of population is killed (LT90) is highest in potato rot nematode adults (−7.4°C) and lower in J4 and younger juveniles (−9.4 and −14.5°C, respectively). Evidently, the most cold-resistant stages for *D. destructor* are the younger juveniles and not J4 as is the case for *D. dipsaci* (Bosher & McKeen, 1954). There is a great variation between survival ability of younger juveniles as shown by LT90 upper and lower fiducial limits (−11.07

Table 1. *Effect of the media on mortality of* Ditylenchus destructor *post-eclosion stages after 24 h exposure to* $-5 \pm 1°C$.

	Water	Gravel	Potato	M9 buffer
No. replications	8	5	5	11
Range total no. per sample (min/max)	10/30	10/10	86/288	10/40
Range no. dead per sample (min/max)	9/30	7/10	30/70	2/10
Total no. dead/total no.	136/140	47/50	275/867	85/273
Total mortality ± SE (%)	97.1 ± 1.4^{a}	94.0 ± 3.4^{a}	31.7 ± 1.6^{b}	31.1 ± 2.8^{b}

Mortalities followed by different letters are statistically significantly different ($P < 0.05$).

Table 2. *Mortality of post-eclosion stages of* Ditylenchus destructor *after 24 h exposure to various temperatures.*

Age group	No. dead/total no. (mortality ± SE, %)				
	22°C	−5°C	−8°C	−18°C	−30°C
J2 + 3	$15/50\ (30.0 \pm 6.5)^{A}$	$85/265\ (32.1 \pm 2.9)^{a,A}$	$821/1091\ (75.3 \pm 1.3)^{a,B}$	$237/268\ (88.4 \pm 2.0)^{a,C}$	$88/90\ (97.8 \pm 1.6)^{D}$
J4	$11/40\ (27.5 \pm 7.2)^{A}$	$78/177\ (44.1 \pm 3.7)^{b,A}$	$430/488\ (88.1 \pm 1.5)^{b,B}$	$242/245\ (98.8 \pm 0.7)^{b,C}$	$29/29\ (100.0 \pm 0.0)^{C}$
Adult	$7/28\ (25.0 \pm 8.3)^{A}$	$53/142\ (37.3 \pm 4.1)^{ab,A}$	$358/367\ (97.5 \pm 0.8)^{c,B}$	$179/179\ (100.0 \pm 0.0)^{b,B}$	$11/11\ (100.0 \pm 0.0)^{B}$

Mortalities followed by different lowercase letters within a column and different capital letters within a row are statistically significantly different ($P < 0.05$).

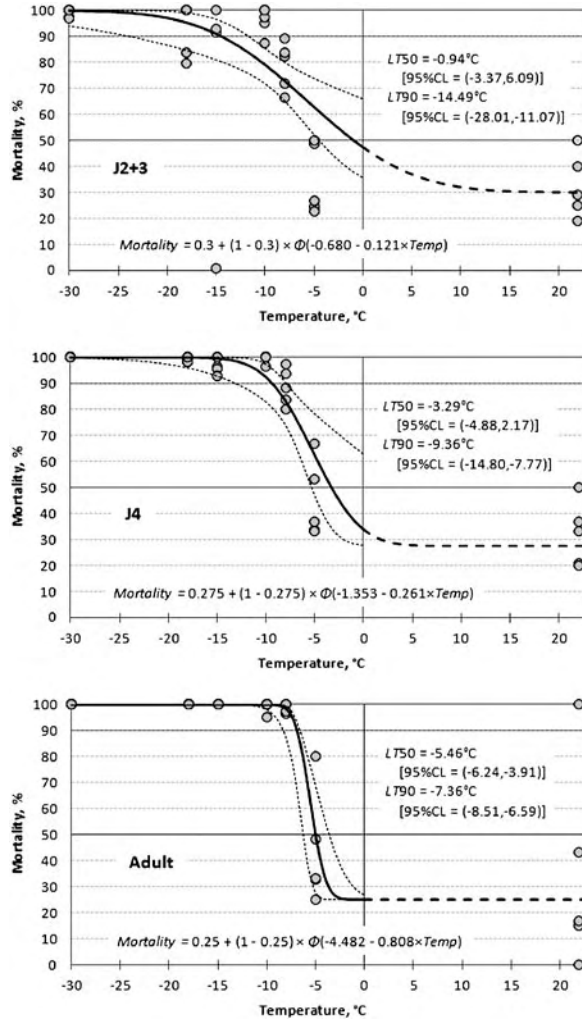

Fig. 1. *Cold tolerance of post-eclosion stages of* Ditylenchus destructor *after 24 h exposure to temperature regimes from −5 to −30°C. Circles mark the mortality of different replicates, solid line (dashed line for positive temperatures) corresponds to the probit model corrected for control mortality at 22°C using Abbott's formula and dotted lines are the 95% confidence limits of prediction.*

and −28.0°C). On the other hand, LT50 was highest in younger juveniles (−0.94°C) and lowest in adults (−5.46°C).

Despite differences in media and experimental methods, the LT50 for a *D. dipsaci* population consisting predominately of J4, measured in an earlier study (Smith *et al.*, 2008), falls within the confidence limits of our results with *D. destructor* J4 and adults at around −4°C.

The soil temperature records of the Rõhu automatic weather station of the Estonian University of Life Sciences in the vicinity of Tartu (58°17′N, 26°18′E) have shown that even during the harshest winter seasons of recent years soil temperatures rarely fell below zero, with sensors buried in grassland at a depth of 10 cm (EMU, 2010). Moreover, Sutinen *et al.* (1999) simulated soil temperatures at a depth of 5 cm in Finnish Lapland (67°30′N, 29°30′E), on the basis of the air temperature and precipitation data and have shown that subzero soil temperatures would hardly occur in mild winters (min. −2.2°C with no precipitation) or moderate winters with high or mean precipitation levels (around 0°C). In the case of high and mean precipitation in a harsh winter, the soil temperature stayed above −5°C after snow accumulation started. The model was based on Finnish meteorological recordings, indicating that the lowest monthly minimum air temperatures in 30 years were −50°C in January; this has never occurred in Estonia with its more southerly location. In northern European countries, invertebrates may experience different conditions in different years; winters with a deep snow layer may alternate with snowless winters and temperatures may vary widely (Hiiesaar *et al.*, 2009). Hence, we may conclude that only the harshest winters with no permanent snow cover are able to lower the soil temperature to below −5°C, *i.e.*, the temperature point critical for suppressing populations of *D. destructor* in northern temperate areas.

The results of our first experiment confirm that the most favourable media for surviving moderate freezing are host plant tissues and M9 buffer. Free-living soil and plant nematodes are adapted to micro-environments with relatively high concentrations of electrolytic compounds (De Ley & Mundo-Ocampo, 2004), which prevent ice crystal formation in the buffer solution. Likewise, host plant tissues inhibit formation of inoculative ice crystals, since ice first forms in the dilute apoplastic solution by extracellular freezing. This keeps the nematodes surrounded with ice while protecting them from desiccation. Cold-induced starch-hexose conversion results in higher concentrations of glucose and its metabolites (Sowokinos, 2001) in potato tissue. These chemicals are needed by cells to synthesise trehalose, a stress protectant that interacts with, and directly protects, lipid membranes from the damage caused by environmental stresses such as desiccation and freezing (Behm, 1997). However, there is no direct evidence that potato starch metabolites contribute to the trehalose pool in nematodes.

We found the liquid media unsuitable for cold tolerance experiments in more severe cold due to ice formation. Hand picking would guarantee the provision of an exact number of viable individuals in a replication before the onset of the experiment; however, additional manipulation stress leads to an overestimation of the mortality. Likewise, overestimation of the mortality can be assumed in the case of the infested potato tissue standard samples as the population is sampled at random and a certain number of dead individuals may already be present regardless of the cold exposure. Our results have shown that the host plant tissue samples still give the most conservative mortality estimate, which is why we decided in favour of this method for future experiments.

It has been suggested that nematodes may desiccate rather than freeze in soil due to freeze concentration of nematode body fluids (Pickup, 1990; Forge & MacGuidwin, 1992). This may prevent the nematode from freezing. However, excessive concentration of extracellular and intracellular electrolytes may damage the cell membrane, leading to cytolysis (Mazur, 2004; Muldrew *et al*., 2004). Excessive osmotic shrinkage (Meryman, 1968) or membrane destabilisation (Steponkus & Lynch, 1989) may also occur, causing cryo-injury from which the cells cannot recover. Without contact with surface water, and yet not desiccated, a nematode could employ cryo-protective dehydration (Wharton *et al*., 2003). Holmstrup and Westh (1995) suggest that a supercooled animal surrounded with ice will lose water and desiccate because the vapour pressure of supercooled solution is higher than that of ice. Nevertheless, unlike *D. dipsaci*, *D. destructor* is unable to withstand excessive desiccation.

Nematodes may be able to prevent inoculative freezing by the presence of protective structures such as eggshell (Wharton, 1995). This might have been the case for a few surviving J2 at −30°C in our experiment as the number of eggs was not counted and there is a possibility that viable individuals hatched after the termination of freezing and before assessment of mortality. Alternatively, it might be hypothesised that the J2 and J3 exhibited another mechanism of cold survival. For example, Ladygina (1970) observed survival of the onion, clover and strawberry races

of *D. dipsaci* and potato stem nematode *D. destructor* in pieces of fresh host material after cooling those in cryostat down to −28/−29°C for several hours, but a few specimens remained viable after 5 min exposure of the infested plants to liquid nitrogen (−196°C). Depending on the cooling rate, vitrification of the body fluids may occur in such conditions, which allows cryopreservation because water, ions and other solutes remain in their original intra- and extracellular compartments, and damage due to freeze concentration and mechanical distortion is avoided (Lee, 2010). However, since natural cooling rates do not exceed 1-2°C h^{-1} (Steffen *et al*., 1989), vitrification is an unlikely strategy for body water management in overwintering plant and soil nematodes. On the other hand, we have proved that, due to the facultative endoparasitic nature of the species, host plant tissues may lower, but not prevent, reduction of population density in subzero temperatures.

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VI

Snow manipulation as an alternative nematode control strategy

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Abstract

Within the limitations of restricted choice of nematicides, research attempts shall be directed to developing alternative nematode control strategies. A correlative study was conducted to compare the effect of snow manipulations over the soil temperature with the laboratory results of potato rot nematode (*Ditylenchus destructor* Thorne) survival. Soil temperature was measured by data loggers buried in 5 cm soil under thick snowpack, densified snow base and under the bare ground from where the snow was removed. The results confirm the suggestion that the natural snow cover isolates the ground surface from the negative temperature extremes, and the consequent survival of potato rot nematodes is not likely to be reduced. On the other hand, snow densification prior to the arrival of a cold front resulted in a significant temperature drop down to −2ºC, which may cause more than 50% mortality of the most temperature sensitive age group i.e. younger larvae. The most efficient soil temperature manipulation was snow removal, which may result in 90% mortality of adults and fourth-stage juveniles in exposed soil strata. None of the tested treatments were able to guarantee 100% mortality of any of the life stages involved in the comparison. We suppose the methods are appropriate for further efficiency testing in heavily infested fields, especially in small scale and ecologically sustainable agriculture.

Key words: soil temperature, snowpack densification, snow removal, *Ditylenchus destructor*, survival.

Introduction

Potato rot nematode, *Ditylenchus destructor* Thorne is a polyphagous pest known to have caused occasionally serious economic damages, especially, in Eastern European potato production. *Ditylenchus* hatch from eggs as vermiform second-stage juveniles (J2), since the first molt precedes eclosion. The juvenile grows and undergoes three more molts (J3, J4 and adult) to become a mature male or female. Within the limitations of restricted choice of nematicides available in Europe (none of which are currently registered in Estonia), nematode control efforts have to cope mostly with alternative strategies even in intensive agriculture. Because potato rot nematode is capable of remaining viable in soil (Svilponis et al., 2008), presence of economically significant contamination in the field depends of a great extent on the level of overwintering survival. Weed suppression has been reported as one of the key factors to potato rot nematode control (Andersson, 1971). However, in modern farming, there are rising concerns about the effect of weed control regimes on biodiversity, as higher biodiversity usually confers greater ecosystem stability. Hence, rather than eliminating species, task is to find methods to restore and maintain natural balance within the cropping system.

The geographic range of pest populations are limited mainly by the availability of host plants and the suitability of the climatic conditions. The extent to which pest managers can employ negative temperature extremes to protect the crop depends upon their ability to manipulate exposure of pests typically to naturally occurring cold temperatures by removing insulation (Hoy, 1997).

Snow is well known for its low thermal conductivity insulating underlying soil, with significant effect for physical and biological processes. Even though, there are no studies of spatial variability in snow cover and the winter soil environment as related to potato rot nematode overwintering mortality, Milner et al. (1992) have shown a successful attempt to manage Colorado potato beetle, *Leptinotarsa decemlineata* Say using an artificially mulched trap crop. In the 0-15 cm soil strata, where most of the adult beetles overwintered, after the removal of mulch and snow cover, temperatures dropped from 0 to −11.7°C, whereas in undisturbed plots, temperature remained close to 0°C. Adult survival was significantly higher in snow-covered, unmulched controls and mulched habitats (=26%) than in disturbed habitats (=7%) (Milner et al., 1992). Even though some earlier work suggest that preventing snow covering the ground has no significant effect on the relative abundance of soil nematodes (Sulkava, Huhta, 2003), others have demonstrated that freezing plays important role both in soil physical properties and community composition, as reviewed by Henry (2007).

Early winter tilling or turning of the soil is well known pest management practice in vegetable gardens, because it exposes over-wintering insects to winter conditions. This has been a recommended control strategy also for limiting root-nematode survival (Heinz, Goellner Mitchum, 2010). For North-West Russia, among other specific pest management measures, snow rolling to reduce soil temperature in the depth of tillering node against snow mold has been recommended in winter cereals (Nikolaev, 2009). In our study, we measured the effect of snowpack densification and snow removal on the soil temperature, in order to estimate the influence of the soil temperature manipulations on the potato rot nematode winter survival.

Materials and methods

The experiment was conducted at the Tõnisson Experimental Garden, Estonian University of Life Sciences, at Eerika near Tartu, Estonia (58º21′ N, 26º40′ E) on sandy loam Stagnic Luvisol in WRB classification (Reintam et al., 2008) from January 13 until April 12, 2011. The test plots were located at the centre of an open field with winter rye as a cover crop (cabbage as previous culture) in a radial arrangement as shown in Figure 1. After the demarcation of the plots and digging out the bare ground under the natural snowpack of 52 cm, the data loggers (LogTag Model: TRIX-8 Temp. Logger, Micro *DAQ*.com, China), isolated from the soil humidity, were buried in 5 cm soil as it has been demonstrated that *Ditylenchus dipsaci,* a species related to *D. destructor*, is most abundant in crown soil of the host plant (Simmons et al., 2008).

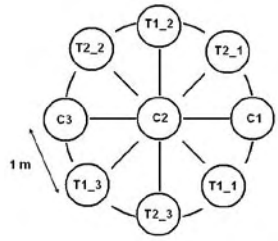


Figure 1. Arrangement of control plots (C) and test plots (T1 and T2) in three replications for the soil temperature manipulation experiment, with T1 marking the snow removal treatments and T2 the snow densification treatments

The snow cover was carefully restored in control and snow densification treatments. To increase density and reduce the isolation capacity of the snow, 50 cm diameter plots were trampled

down by boots into a flat solid base, which measured 17-21 cm in depth. To test the effect of reduced snowpack thickness, snow was completely removed on 50 cm diameter plots. Even when the snowpack of the control plots was compacted during the onset of the experiment because of unavoidable disturbances while the data loggers were set in their places, the original snow cover thickness was quickly restored by snowfall and wind, which resulted in 60-63 cm deep snowpack by January 26, when the natural undisturbed snow layer measured 63 cm. To keep the fresh snow accumulation on treated plots at less than 10 cm level by the arrival of a cold front, the snow treatments were carried out on 19, 24 and 26 January and 14 February. The arrival of the cold front was recorded on 12 February, when the mean daily air temperatures fell below -10ºC (min. −17.2ºC) and it lasted until 25 February, when the mean temperature was −13.2ºC (min. −21.5ºC). Measurements were programmed to be recorded automatically every 30 minutes. The data loggers were excavated and the measurements retrieved in spring after the snow melt and daily average temperature rose above 0.5 ºC. The effect of snow manipulations was compared by repeated measures ANOVA, for the period after the last snow treatment on 14 February until the passing of the cold front. Because of the ethical and licensing considerations, the experimental garden was not contaminated with infective nematodes but the temperature records were compared with the results of an earlier laboratory experiment.

Results and discussion

Soil temperature readings as well as the mean air temperatures and daily precipitation are presented in Figure 2. There was a statistically significant difference in mean soil temperatures between the treatments ($F_{4, 142} = 498.7$, $p < 0.001$), with the significant interaction of date and temperature ($F_{44, 142} = 1.49$, $p = 0.04$). Fisher LSD analysis revealed that snow removal treatment was significantly different from the other manipulations in all the compared measurement points, while the differences between the control and the soil densification treatment appeared only after February, 19. The results were compared with our earlier experiment on cold tolerance of potato rot nematode post-eclosion stages, which had been kept in subzero temperature regimes for 24 h. The mortality estimations for 50 and 90% of population of the nematode are given in Table.

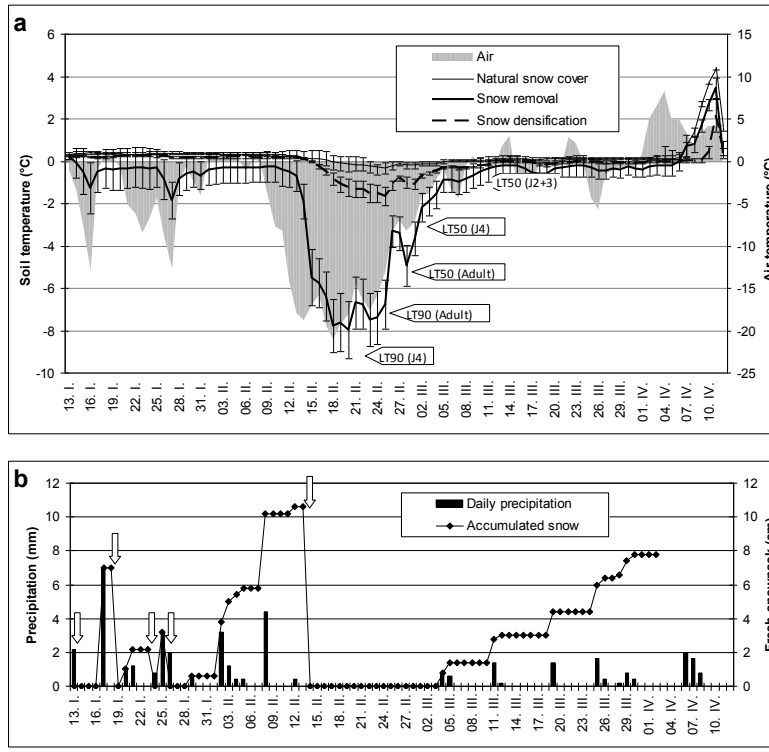
Table. Probit model for mortality and lethal temperatures (*LT*50 and *LT*90) with confidence limits (95% *CL*) for development stages of *Ditylenchus destructor* after exposure to subzero temperatures for 24 h

Age group	Model equation* *Mortality* =	*LT*50, °C (95% *CL*)	*LT*90, °C (95% *CL*)
J2 + 3	$0.3 + 0.7 \times \Phi(-0.680 - 0.121 \times temp)$	−0.94 (−3.37, 6.09)	−14.49 (−28.01, −11.07)
J4	$0.275 + 0.725 \times \Phi(-1.353 - 0.261 \times temp)$	−3.29 (−4.88, 2.17)	−9.36 (−14.80, −7.77)
Adult	$0.25 + 0.75 \times \Phi(-4.482 - 0.808 \times temp)$	−5.46 (−6.24, 3.91)	−7.36 (−8.51, −6.57)

Notes. The data on younger juveniles (second- and third-stage juveniles, J2 and J3) is combined. Adapted from Švilponis et al. (2011). Φ – normal cumulative distribution function; * – probit model and lethal temperatures were corrected for control mortality at 22°C using Abbott's formula

While the minimal subzero temperatures during the passage of the cold front in control did not exceed −0.8°C, the snow densification and snow removal manipulations resulted in minimum temperatures of −2.1°C and −10.3°C respectively. In another experiment lasting from September 29, 2010 until May, 2011, the surface temperatures under undisturbed natural snow cover in Tartu

ranged at lowest between +0.7°C and −0.2°C with subzero temperatures lasting from February 22 to March 02 (Hiiesaar et al., unpublished data). This indicates that lethal temperatures for mortality of 50% of the younger juvenile stages of potato rot nematodes may be obtained by snow densification but more laborious snow removal may result in soil temperature drop to the level necessary for 90% mortality of older juveniles and adults, even though lower lethal temperature for adults (−15°C) was not achieved.



Note. Block arrows indicate lethal temperatures for 50 (LT50) and 90% mortality (LT90) of post-eclosion stages (adults, fourth-stage juveniles J4 and combined second- and third-stage juveniles J2 + 3) of potato rot nematode in 24 h exposition to subzero temperatures (according to Svilponis et al., 2011). (b) Mean daily precipitation (according to the Laboratory of Environmental Physics, http://meteo.physic.ut.ee) and accumulated snow cover thickness (calculated roughly by the rule: 1mm precipitation = 1cm snow). Block arrows denote the dates of snow treatment.

Figure 2. (a) The effect of a snow treatment on mean (±SD) soil temperatures at 5 cm depth

The deeper the soil, the higher the temperatures. Some nematodes have been shown to be able for seasonal vertical distribution fluctuations. The natural populations of Heterorhabditid and Steinernematid nematodes migrated to deeper layers during summer, presumably to avoid the unfavourable environmental conditions caused by high temperatures or lack of humidity (Garcia Del Pino, Palomo, 1997). Studies examining temperature gradient fluctuations have indicated that differences in the rate of thermal adaptation by a nematode can reverse the net direction it moves vertically in response to soil surface heating and cooling (Dusenbery, 1988). When movement of *Ditylenchus phyllobius*, *Steinernema glaseri* and *Heterorhabditis bacteriophora* was largely random relative to the thermal surface, *Rotylenchulus reniformis* and *Meloidogyne incognita* oriented towards it (Robinson, 1994). The oriented locomotion of plant parasitic nematodes in response to thermal and moisture gradients in winter is to be studied in more details.

Overwintering survival of the soil dwelling pests is determined not only by cold tolerance and ability for vertical migration but also their resistance to dehydration. Benoit et al. (1988) have shown that water accumulated in frozen soil layers by migration to the freezing point. The result was less moisture at deeper soil depths. By inducing soil freezing, this may influence potato rot nematode survival indirectly, since the deeper soil moisture will be reduced beyond optimal limits, which possibly damages the individuals dwelling beyond the freezing zone. *Ditylenchus destructor* is very susceptible to desiccation, in contrast to their related species *D. dipsaci*, a pest sometimes inhabiting aerial parts of host plants where they may experience high rates of water loss and who is capable of anhydrobiosis (Perry, 1977). Desiccation may be an important stress responsible also for mortality of *Meloidogyne hapla* second-stage juveniles in frozen conditions (Forge, MacGuidwin, 1992). In contrast, snow accumulation has been shown to increase gravimetric soil moisture and associates with a decline in abundance of nematodes typically found in dry soils but an increase in nematodes associated with moist soil (Ayres et al.2010). Since potato rot nematodes are only important in moist conditions, we may assume that large scale snow removal has probably stronger effect on potato rot nematodes than can be concluded from our initial soil temperature manipulation experiment.

As our snow compaction test was begun on a relatively warm day (air temperature +0.8ºC), on slightly molten multi-layered snow structure, optimal final density was not obtained. After removing of the snowpack, burying the data loggers and returning the composite snow aggregates, the treatment effected in reducing the snowpack thickness only for 68% (from 52,6 cm to 17 cm). The resulting soil temperatures were too high to cause significant winter mortality in all but at the LT50 (J2 + 3) level. Thermal conductivity of the compacted snow could be considerably improved if snow-treatment would be initiated early in the season while the snowpack is still lower than 10 cm, or in subzero temperatures, when snow crystals would break instead of sublimation.

One possibility for further improving the effect of soil temperature manipulation is by the use of mulch or ground cover as recommended by Hoy (1997). Since soil under mulch remains at higher temperatures than bare soil, a rapid temperature drop can be provoked if the snow and mulch would get removed immediately before the arrival of the cold front (Milner et al., 1992). The faster the temperature drops, the smaller the possibilities that pests would have a chance for cold hardening or acclimatory changes of the basic cell structural components (Koštal, 2010). However, because the labour costs for removing the mulch are high as it can not be easily raked off when frozen and snow-covered, this strategy will probably be feasible only in limited size fields (Hoy, 1997). In order to elaborate more conclusive results for integrated pest management strategies, this correlative study will be continued as a series of field experiments in collaboration with farms exposed to potato rot nematode infestation.

Conclusions

1. A sufficient proportion of nematodes survive winter conditions in potato-growing areas of Northern Europe to produce economically significant infestations, despite of the rigors of the winter soil environment. Natural snow cover guarantees temperature conditions adequate for potato rot nematode overwintering.

2. Snow cover thickness and density manipulations resulted in significant reduction of temperatures in the upper soil layer during a cold front. The most effective treatment was snow removal, by which temperatures below -10.3°C were achieved. This is less than required to guarantee the mortality of the 50% of all the post-eclosion stages of *D. destructor* as well as 90% of adults, which may considerably affect the winter survival of potato rot nematode population.

3. Snow densification by trampling in smaller areas or snow-rolling in larger fields as well as snow removal can be perspective methods for reducing nematode population densities in heavily infested soils. The novel plant protection techniques may be suitable for practicing in sustainable agriculture.

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2008	Tartu Ülikooli haridusteaduskonna treeningprogramm "Kõrgkoolididaktika"

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Hiiesaar, K.; Metspalu, L.; Jõgar, K.; **Svilponis, E.**; Ploomi, A.; Kivimägi, I. (2009). NeemAzal T/S mõju kartulimardika (*Leptinotarsa decemlineata* Say) käitumisele. [Influence of NeemAzal T/S on the behaviour of Colorado potato beetle (*Leptinotarsa decemlineata* Say)] Teaduskonverentsi "Agronoomia 2009" kogumik (192 - 197). Jõgeva: Paar OÜ

Svilponis, Eha; Kurguzova, Olga (2006). *Phytophthora ramorum* esmaleiud Eestis [First findings of *Phytophthora ramorum* in Estonia]. Agronoomia. EPMÜ Teadustööde Kogumik, 208 - 211.

Svilponis, Eha (2005). *Liriomyza* perekonda kuuluvate ohtlike taimekahjustajate introduktsiooni tõenäosus. [Introduction potential of harmful pests of genus *Liriomyza*]. Agronoomia. EPMÜ Teadustööde Kogumik, 195 - 197.

5.2. Conference abstracts that do not belong to section 5.1:

Švilponis, E.; Hiiesaar, K.; Mänd, M. (2010). Low temperature survival of *Ditylenchus destructor* Thorne (Tylenchida: Anguinidae). Proceedings of the 30th International Symposium of the European Society of Nematologists, Vienna, September 19-23, 2010 (129). IOS Press.

Hiiesaar, K.; **Svilponis, E.**; Metspalu, L.; Jõgar, K.; Mänd, M.; Luik, A.; Karise, R. (2009). Influence of Neem-Azal T/S on feeding activity of *Leptinotarsa decemlineata* (Say). NJF Report, NJF Seminar 422 "Fostering healthy food systems through organic agriculture - Focus on Nordic-Baltic Region", Tartu, Estonia, 25-27 August 2009 (54). Eesti Loodusfoto.

Kivimägi, I.; Ploomi, A.; Metspalu, L.; **Svilponis, E.**; Jõgar, K.; Hiiesaar, K.; Luik, A.; Sibul, I.; Kuusik, A. (2009). Physiology of carabid beetle *Platynus assimilis*. NJF Report, NJF Seminar 422. International Scientific Conference "Fostering healthy food systems through organic agriculture - Focus on Nordic-Baltic Region", Tartu, Estonia, 25-27 August 2009 (54). Eesti Loodusfoto.

Jõgar, K.; Metspalu, L.; Hiiesaar, K.; Ploomi, A.; **Svilponis, E.**; Kuusik, A.; Kivimägi, I.; Luik, A. (2009). Influence of white cabbage cultivars on oviposition preference of the *Pieris rapae* L. NJF Report. NJF Seminar 422. International Scientific Conference "Fostering healthy food systems through organic agriculture - Focus on Nordic-Baltic Region", Tartu, Estonia, 25-27 August 2009 (41). Eesti Loodusfoto.

Metspalu, L.; Hiiesaar, K.; Jõgar, K.; **Svilponis, E.**; Ploomi, A.; Kivimägi, I.; Luik, A.; Mens`hikova, N. (2009). Oviposition preference of Pieris brassicae on different Brassica oleracea cultivars. NJF Report. NJF Seminar 422. International Scientific Conference "Fostering healthy food systems through organic agriculture - Focus on Nordic-Baltic Region", Tartu, Estonia, 25-27 August 2009 (41). Eesti Loodusfoto.

Ploomi, A.; Jõgar, K.; Metspalu, L.; Hiiesaar, K.; **Svilponis, E.**; Kivimägi, I.; Luik, A.; Sibul, I.; Kuusik, A. (2009). Effect of cultivar on oviposition preference of the cabbage moth, *Mamestra brassicae* L. NJF Report. NJF Seminar 422. International Scientific Conference "Fostering healthy food systems through organic agriculture - Focus on Nordic-Baltic Region", Tartu, Estonia, 25-27 August 2009 (40). Eesti Loodusfoto.

6.3. Popular Science articles:

Švilponis, E. (2010). Nematoodid keldris ja aiamaal. [Nematodes in cellars and gardens]. Maakodu, 64 - 65.

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VIIS VIIMAST KAITSMIST

MARIS HORDO

APPLICATION OF DENDROCLIMATOLOGICAL METHODS FOR FOREST GROWTH MODELLING.

DENDROKLIMATOLOOGILISTE MEETODITE KASUTAMINE PUISTU KASVUKÄIGU MODELLEERIMISEL.

Prof. **Andres Kiviste**, dr. **Helena Henttonen**, dr. **Samuli Helama**

27. aprill 2011

MARIT KOMENDANT

ANTENNAL CONTACT CHEMORECEPTION IN GROUND BEETLES (COLEOPTERA: CARABIDAE).

JOOKSIKLASTE (COLEOPTERA: CARABIDAE) ANTENNAALNE KEMORETSEPTSIOON.

Vanemteadur **Enno Merivee**, prof. **Anne Luik**

6. mai 2011

VAHUR PÕDER

COMPATIBILITY OF ENERGY CONSUMPTION WITH THE CAPACITY OF WIND GENERATORS.

ENERGIA TARBIMISE SOBIVUS TUULEGENERAATORITE VÕIMSUSEGA.

Prof. **Andres Annuk**

21. juuni 2011

MERIKE LILLENBERG

RESIDUES OF SOME PHARMACEUTICALS IN SEWAGE SLUDGE IN ESTONIA, THEIR STABILITY IN THE ENVIRONMENT AND ACCUMULATION INTO FOOD PLANTS VIA FERTILIZING.

MÕNEDE RAVIMIJÄÄKIDE SISALDUS EESTI REOVEESETTES, NENDE STABIILSUS KESKKONNAS JA AKUMULEERUMINE KOMPOSTVÄETISEST TOIDUTAIMEDESSE.

Prof. **Lembit Nei**, prof. **Kalev Sepp**

16. september 2011

MARGE MALBE

THE ROLE OF SELENIUM IN UDDER HEALTH OF DAIRY COWS.

SELEENI TOIME LÜPSILEHMADE UDARA TERVISELE

Prof. emer. **Hannu Saloniemi**, dots. **Andres Aland**

7. oktoober 2011

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